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(71) Applicants: BRESATEC LTD. [AU/AU]; P.O. Box 11, Rundle Mall, Adelaide, S.A. 5000 (AU). ST. VINCENT'S HOSPITAL (MELBOURNE) LIMITED [AU/AU]; 41 Victoria Parade, Fitzroy, VIC 3065 (AU).			
(72) Inventors: PEARSE, Martin, J.; 37 Chute Street, Mordialloc, VIC 3195 (AU). CRAWFORD, Robert, J.; 7 Mirani Court, West Lakes Shore, S.A. 5020 (AU). ROBBINS, Allan, J.; Lot 8, Barker Road, Waterloo Corner, S.A. 5110 (AU). RATHJEN, Peter, D.; 1 Mimosa Avenue, Blackwood, S.A. 5051 (AU). d'APICE, Anthony, J., F.; 988 Burke Road, Balwyn, VIC 3103 (AU).			
(74) Agent: MAXWELL, Peter, Francis; Peter Maxwell & Associates, Blaxland House, 5 Ross Street, North Parramatta, NSW 2151 (AU).			<b>Published</b> <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: MATERIALS AND METHODS FOR MANAGEMENT OF HYPERACUTE REJECTION IN HUMAN XENOTRANSPLANTATION			
(57) Abstract <p>Human pre-formed xenoantibodies play an important role in the hyperacute rejection response in human xenotransplantation. Disclosed are materials and methods for removing or neutralizing such antibodies. Also disclosed are materials and methods for reducing or eliminating the epitopes in the donor organs that are recognized by such antibodies. Such epitopes are formed as the result of activity by the enzyme <math>\alpha</math>-1,3 galactosyltransferase. The porcine gene encoding <math>\alpha</math>-1,3 galactosyltransferase is disclosed, as are materials and methods for inactivating ("knocking out") the <math>\alpha</math>-1,3 galactosyltransferase gene in mammalian cells and embryos. Included are nucleic acid constructs useful for inactivating the <math>\alpha</math>-1,3 galactosyltransferase gene in a target cell. Also disclosed is a novel leukemia inhibitory factor (T-LIF) that is useful for maintenance of embryonic stem cells and primordial germ cells in culture.</p>			

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**MATERIALS AND METHODS FOR MANAGEMENT OF HYPERACUTE  
REJECTION IN HUMAN XENOTRANSPLANTATION**

Field of the Invention

5           This invention relates generally to the field  
of xenotransplantation. In particular this invention  
relates to methods and materials for reduction or  
elimination of the hyperacute rejection response in  
humans. More particularly, this invention relates to  
10 methods for treating human serum to reduce or eliminate  
hyperacute rejection. This invention also relates to  
methods and materials for generating non-human organs  
lacking or having reduced  $\alpha$  1,3 galactosyl transferase  
activity.

15                           Background of the Invention

It is widely acknowledged that there is an acute,  
worldwide shortage of human organs for transplantation.  
This is in spite of legislative changes and education  
programs to increase public awareness of the problem. In  
20 the United States, for example, there is an estimated  
annual shortfall of approximately 18,000 kidneys/year.  
Similarly, in Australia in 1992, only 41% of renal  
patients awaiting transplantation received transplants.  
In Japan the imbalance between supply and demand is even  
25 greater due to religious prohibitions on the use of  
organs from cadaveric donors.

The benefits of transplantation can be seen by  
comparing the rehabilitation rates of transplant patients  
with those of dialysis patients. In Australia and New  
30 Zealand, the majority of transplant patients (60%) are  
capable of full time work or school with a further 10% in  
part time work, while only 7% are unfit for work. In

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contrast, 23% of dialysis patients are capable of full time work or school, with 15% involved in part time work and 20% unfit for work. The remainder are "retired." Fifteenth Report of the Australia and New Zealand  
5 Dialysis and Transplant Registry (ANZDATA), Queen Elizabeth Hospital, Woodville, S.A., APS Disney, ed. (1992).

The direct financial cost of dialysis in Australia and New Zealand is approximately \$A45,000/patient/year.  
10 In addition, indirect costs due to unemployment and sickness are higher in dialysis patients and the social costs are considerable. Transplantation engenders an expense of approximately \$A30,000/patient in the first year and \$A14,000/patient/year thereafter. These  
15 statistics indicate that a) transplantation is the optimal therapy for end stage renal failure; b) there is an undersupply of donor kidneys; and c) present strategies aimed at increasing the transplant rate have been less than successful. There are, in addition,  
20 serious shortages of other transplantable organs including hearts, livers, lungs and pancreases.

The use of xenografts (transplants between species) is one option for overcoming the short supply of human organs for transplantation. Non-viable, non-  
25 antigenic xenografts are commonly used in vascular reconstruction (bovine arteries) and in cardiac surgery (porcine cardiac valves). However, despite their occasional use in the past, immunological barriers have prevented the common use of viable xenografts. Between  
30 1964 and 1991 a total of 27 non-human primate to human organ xenografts was reported; the longest reported patient survival was 9 months. Two liver transplants from baboon to human were recently performed in anticipation that modern immunosuppressive therapies  
35 could cope with the severe rejection problems likely to

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occur in xenotransplantation. To date, the course of one of these patients has been reported, and in this case rejection was not the direct cause of death. Starzl et al., Baboon-to-Human Liver Transplantation. Lancet 341: 5 65-71 (1993). This clinical experience indicates that a) non-human organs can function and support human life; b) rejection episodes can be reversed by conventional anti-rejection therapy; and c) the mechanisms of rejection are similar, in principle, to those in allograft rejection.

10 It is unlikely that primates will be a satisfactory source of organs for xenotransplantation. Most are endangered species, breed slowly in the wild and poorly in captivity. The baboon is an exception to these generalizations, but other disadvantages limit the  
15 usefulness of this species. Baboons have single pregnancies, long gestation times, are difficult and expensive to maintain and may be infected with or carry organisms, particularly viruses, that are pathogenic in humans. For hearts and kidneys where organ size may be a  
20 consideration, the smaller primates are unsatisfactory as donors to human adults. Finally, the use of primates is likely to arouse considerable opposition from the public.

These difficulties have led to renewed interest in the use of non-primate species as organ donors for human  
25 patients. The pig is a widely acknowledged choice for xenotransplantation into humans. The pig erythrocyte diameter ( $6.5\mu\text{m}$ ) and, by implication, its capillary size, are similar to humans, facilitating connection of xenografts to the human circulatory system. The pig  
30 breeds well in captivity, has a short gestation time and produces large litters. In addition, pigs can be bred and maintained in low pathogen facilities, can be reared to any size and do not arouse the level of public reaction associated with primates.

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The immunological barriers to use of pig organs in human patients include a) an immediate severe ("hyperacute") rejection phenomenon that develops in minutes to hours after transplantation, and b) a proposed acute rejection that develops in days to weeks. Once the hyperacute rejection phenomenon has been overcome, it is expected that normal acute rejection would ensue. This form of rejection is thought to be similar to that experienced with allografts (transplants between individuals of the same species) and should be amenable to normal immunosuppressive therapies.

Both preformed "natural antibodies" (xenoantibodies) and complement regulating factors in human serum are thought to be involved in the process of hyperacute rejection. Hyperacute rejection is thought to be initiated when xenoantibodies bind to epitopes on the endothelium of a donor organ, activating the classical complement pathway.

#### Summary of the Invention

A purified and isolated nucleic acid molecule of the present invention comprises the porcine nucleic acid sequence depicted in Figure 4 (SEQ ID NO: 7), which encodes a porcine polypeptide having  $\alpha$ -1,3 galactosyltransferase activity. Variations on this sequence that may be routinely generated by the skilled artisan include those sequences corresponding to Figure 4 but varying within the scope of the degeneracy of the genetic code. That is, the present invention includes variants of the sequence set out in Figure 4, readily determined by the skilled artisan, that code for the same amino acid sequence encoded by the sequence set out in Figure 4. The present invention also includes a purified and isolated nucleic acid molecule that encodes a porcine  $\alpha$ -1,3 galactosyltransferase and that hybridizes

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under standard high stringency conditions with a sequence complementary to the sequence set out in Figure 4, or with a sequence complementary to a variation of the sequence set out in Figure 4 within the scope of the degeneracy of the genetic code. The complementary strands to the above-described nucleic acid sequences are readily determined by standard methods, and are also within the scope of the present invention.

Within the parameters set out in the preceding paragraph, the present invention includes variants of the porcine  $\alpha$ -1,3 galactosyltransferase coding sequence that preserve the functional characteristics of the native gene product. Such variants include, for example, minor nucleotide variations in the 5' untranslated region or in various coding regions of the disclosed sequence. Minor amino acid variations deriving from changes in the coding regions, that leave a functional  $\alpha$ -1,3 galactosyltransferase catalytic site, membrane anchor domain and stem region as described below, are within the scope of the present invention. Such routine variations in nucleic acid and amino acid sequences can be identified by those having ordinary skill in the art based on the sequence and structural information provided herein.

As used herein, "high stringency conditions" are those hybridization conditions generally understood by the skilled artisan to reflect standard conditions of high stringency as set out in widely recognized protocols for nucleic acid hybridization. See, e.g., Sambrook et al, Molecular Cloning: A Laboratory Manual (2nd Edition), Cold Spring Harbor Laboratory Press (1989), pp. 1.101 - 1.104; 9.47 - 9.58 and 11.45 - 11.57. Generally, these conditions reflect at least one wash of the hybridization membrane in 0.05x to 0.5x SSC with 0.1% SDS at 65°C, or washing conditions of equivalent stringency.

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The present invention also includes a host cell transformed with any of the above-described purified and isolated nucleic acid molecules, as well as a porcine  $\alpha$ -1,3 galactosyltransferase encoded by such transforming nucleic acid molecules and expressed from the host cell. Methods for transforming appropriate host cells and for expressing polypeptides from such host cells are known in the art and are described, for example, in Sambrook et al., (1984), pp. 12.2-12.44; 16.3-17.44.

- 10       The invention further includes a DNA construct useful for inactivating the porcine  $\alpha$ -1,3 galactosyltransferase gene by insertion of a desired DNA sequence into an insertion site of the gene. As used herein, the term " $\alpha$ -1,3 galactosyltransferase gene"
- 15 includes the exons encoding or potentially encoding  $\alpha$ -1,3 galactosyltransferase, introns contiguous with such exons, and regulatory elements associated with such exons and introns. The DNA construct includes the desired DNA sequence flanked by first and second homology sequences.
- 20 These first and second homology sequences are sufficiently homologous, respectively, to first and second genomic sequences flanking the insertion site to allow for homologous recombination of the DNA construct with the porcine  $\alpha$ -1,3 galactosyltransferase gene when
- 25 the DNA construct is introduced into a target cell containing the porcine  $\alpha$ -1,3 galactosyltransferase gene. Preferably the insertion site is within exon 4, exon 7, exon 8 or exon 9 of the porcine  $\alpha$ -1,3 galactosyltransferase gene. The desired DNA sequence is
- 30 preferably a selectable marker, including but not limited to the neo<sup>R</sup> gene, the hydromycin resistance (hyg<sup>R</sup>) gene and the thymidine kinase gene. The desired DNA sequence may be bordered at both ends by FRT DNA elements, with stop codons for each of the three reading frames being
- 35 inserted 3' to the desired DNA sequence. Presence of the



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FRT elements allows the selectable marker to be deleted from the targeted cell, and the stop codons ensure that the  $\alpha$ -1,3 galactosyltransferase gene remains inactivated following deletion of the selectable marker.

5           The invention further includes a DNA construct useful for inactivating the murine  $\alpha$ -1,3 galactosyltransferase gene by insertion of a desired DNA sequence into an insertion site of the gene. The DNA construct includes the desired DNA sequence flanked by  
10 first and second homology sequences. These first and second homology sequences are sufficiently homologous, respectively, to first and second genomic sequences flanking the insertion site to allow for homologous recombination of the DNA construct with the murine  $\alpha$ -1,3  
15 galactosyltransferase gene when the DNA construct is introduced into a cell containing the murine  $\alpha$ -1,3 galactosyltransferase gene. Preferably the insertion site is within exon 4, exon 7, exon 8 or exon 9 of the murine  $\alpha$ -1,3 galactosyltransferase gene. The desired  
20 DNA sequence is preferably a selectable marker, including but not limited to the neo<sup>R</sup> gene, the hyg<sup>R</sup> gene and the thymidine kinase gene. The desired DNA sequence may be bordered at both ends by FRT DNA elements, with stop codons for each of the three reading frames being  
25 inserted 3' to the desired DNA sequence. Presence of the FRT elements allows the selectable marker to be deleted from the targeted cell, and the stop codons ensure that the  $\alpha$ -1,3 galactosyltransferase gene remains inactivated following deletion of the selectable marker.

30           The invention also includes methods for generating a mammalian totipotent cell having at least one inactivated (non-functional)  $\alpha$ -1,3 galactosyltransferase allele, where the totipotent cell is derived from a mammalian species in which alleles for the  $\alpha$ -1,3  
35 galactosyltransferase gene normally are present and

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functional. A "functional" allele is capable of being transcribed and translated to produce a polypeptide having an activity the same as or substantially similar to the native  $\alpha$ -1,3 galactosyltransferase. The methods  
5 include providing a plurality of cells characterized as totipotent cells of the aforementioned mammalian species, introducing into the totipotent cells a nucleic acid construct effective for inactivating the  $\alpha$ -1,3 galactosyltransferase gene by insertion of a desired DNA  
10 sequence into an insertion site of the gene through homologous recombination, and then identifying a totipotent cell having at least one inactivated  $\alpha$ -1,3 galactosyltransferase allele.

The totipotent cells can include, without  
15 limitation, embryonic stem (ES) cells, primordial germ cells (PGC's) and eggs. The cells can be taken from a variety of mammalian species in which alleles for the  $\alpha$ -1,3 galactosyltransferase gene are present and functional, including without limitation murine and  
20 porcine species.

The invention further includes methods for generating a mammal lacking a functional  $\alpha$ -1,3 galactosyltransferase gene, where the mammal belongs to a species having a functional  $\alpha$ -1,3 galactosyltransferase  
25 gene. The methods include providing a mammalian totipotent cell having at least one inactivated  $\alpha$ -1,3 galactosyltransferase allele, where the totipotent cell is derived from the aforementioned mammalian species having a functional  $\alpha$ -1,3 galactosyltransferase gene,  
30 manipulating the totipotent cell such that mitotic descendants of the cell constitute all or part of a developing embryo, allowing the embryo to develop to term, recovering a neonate individual derived from the embryo, and raising and breeding the neonate to obtain a  
35 mammal homozygous for an inactivated  $\alpha$ -1,3

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galactosyltransferase alleles, i.e., a mammal in which both  $\alpha$ -1,3 galactosyltransferase allele are inactivated.

The totipotent cells can include, without limitation, ES cells, PGC's and eggs. The cells can be  
5 taken from a variety of mammalian species in which alleles for the  $\alpha$ -1,3 galactosyltransferase gene are present and functional, including without limitation murine and porcine species. ES cells and PGC's are manipulated in various ways such that their mitotic  
10 descendants are found in a developing embryo. These manipulations can include, without limitation, injection into a blastocyst or morula, co-culture with a zona pellucida-disrupted morula, and fusion with an enucleated zygote. Cells injected into a blastocyst- or morula-  
15 stage embryo become incorporated into the inner cell mass of the blastocyst embryo, giving rise to various differentiated cell types of the resulting embryo, including in some cases germ cells. The embryo derived from such manipulations is a chimera composed of normal  
20 embryonic cells as well as mitotic descendants of the introduced ES cells or PGC's. Alternatively, chimeric embryos can be obtained by co-culturing at least one ES cell or PGC with a morula embryo in which the zona pellucida is sufficiently disrupted to allow direct  
25 contact between the ES cell/PGC and at least one cell of the morula. The zona pellucida-disrupted embryo may be an embryo that is completely free of the zona pellucida. Finally, the genome of an ES cell or PGC can be incorporated into an embryo by fusing the ES cell/PGC  
30 with an enucleated zygote. Such a procedure is capable of generating a non-chimeric embryo, i.e., an embryo in which all nuclei are mitotic descendants of the fused ES cell/PGC nucleus. The resulting embryos are implanted in a recipient female, or surrogate mother, and allowed to  
35 develop to term.

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When eggs, as opposed to ES cells or PGC's, are directly injected with a nucleic acid construct effective for inactivating the  $\alpha$ -1,3 galactosyltransferase gene, the eggs can be manipulated to form an embryo by  
5 implanting into a recipient female.

The invention also includes a mammal, produced through human intervention, that lacks a functional  $\alpha$ -1,3 galactosyltransferase gene. The mammal belongs to a species in which the  $\alpha$ -1,3 galactosyltransferase gene is  
10 normally present and functional. The mammal can be, without limitation, a mouse or a pig.

The invention further includes a purified and isolated nucleic acid molecule comprising a nucleic acid sequence selected from the group consisting of (1) the  
15 nucleic acid sequence depicted in Figure 26 (SEQ ID NO: 25), (2) a sequence corresponding to the sequence of (1) within the scope of the degeneracy of the genetic code, and (3) a sequence that encodes murine T-LIF and that hybridizes under standard high stringency conditions with  
20 a sequence complementary to the sequence of (1) or (2). The complementary strands to the above-described nucleic acid sequences are readily determined by standard methods, and are also within the scope of the present invention.

25 The present invention also includes a host cell transformed with any of the purified and isolated nucleic acid molecules described in the preceding paragraph, as well as a T-LIF polypeptide encoded by such transforming nucleic acid molecules and expressed from the host cell.

30 The invention further includes a purified and isolated nucleic acid molecule comprising a nucleic acid sequence selected from the group consisting of (1) the nucleic acid sequence depicted in Figure 27 (SEQ ID NO: 31), (2) a sequence corresponding to the sequence of (1)  
35 within the scope of the degeneracy of the genetic code,

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and (3) a sequence that encodes human T-LIF and that hybridizes under standard high stringency conditions with a sequence complementary to the sequence of (1) or (2). The complementary strands to the above-described nucleic acid sequences are readily determined by standard methods, and are also within the scope of the present invention.

The present invention also includes a host cell transformed with any of the purified and isolated nucleic acid molecules described in the preceding paragraph, as well as a T-LIF polypeptide encoded by such transforming nucleic acid molecules and expressed from the host cell.

The invention further includes a method for eliminating or reducing hyperacute rejection of non-primate mammalian cells by human serum, comprising adding, to the human serum, a physiologically acceptable amount of galactose or a saccharide in which the terminal carbohydrate is an  $\alpha$  galactose linked at position 1, prior to exposure of the human serum to the non-primate cells. The amount of galactose or saccharide added is sufficient to reduce or eliminate the hyperacute rejection response. The saccharide can be, without limitation, melibiose, galactose  $\alpha$ 1-3 galactose or stachyose. Alternatively, the human serum can be treated so as to be substantially depleted of immunoglobulin, IgM antibodies, anti-GAL IgM and IgG antibodies, or anti-GAL IgM antibodies. The invention further includes affinity-treated human serum substantially free of anti-GAL antibodies or of anti-GAL IgM antibodies.

#### 30 BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 is a graphical representation of fluorescence intensity following immunofluorescent staining of porcine aortic endothelial cells with anti-

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GAL antibody alone or with anti-GAL antibody that was preincubated with selected saccharides.

FIGURE 2 shows the results of an experiment in which lysis of porcine aortic endothelial cells by human serum and by purified anti-GAL antibodies was determined using a <sup>51</sup>CR release assay.

FIGURE 3 depicts physiograph tracings of perfused rat heart contractions in the presence of human serum with or without selected saccharides.

FIGURE 4 is a comparison of the porcine  $\alpha$ -1,3 galactosyltransferase cDNA sequence with the corresponding murine and bovine sequences. PGTC D = porcine sequence. BOVGSTA = bovine sequence. MUSGLYTNG = murine sequence.

FIGURE 5 is a comparison of the porcine  $\alpha$ -1,3 galactosyltransferase amino acid sequence with the corresponding murine and bovine amino acid sequences. PGT = porcine sequence. BGT = bovine sequence. MGT = murine sequence.

FIGURE 6 depicts the Sall restriction sites in four overlapping phage clones spanning a portion of the murine  $\alpha$ -1,3 galactosyltransferase genomic region.

FIGURE 7 is a detailed restriction map of murine  $\alpha$ -1,3 galactosyltransferase subclone p $\alpha$ GT-S5.5.

FIGURE 8 is a detailed restriction map of murine  $\alpha$ -1,3 galactosyltransferase subclone p $\alpha$ GT-S4.0.

FIGURE 9 is a detailed restriction map of murine  $\alpha$ -1,3 galactosyltransferase subclone p $\alpha$ GT-S11.

FIGURE 10 is a detailed restriction map of murine  $\alpha$ -1,3 galactosyltransferase subclone p $\alpha$ GT-S13.

FIGURE 11 is an additional detailed restriction map of murine  $\alpha$ -1,3 galactosyltransferase subclone p $\alpha$ GT-S5.5.

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FIGURE 12 is an additional detailed restriction map of murine  $\alpha$ -1,3 galactosyltransferase subclone p $\alpha$ GT-S4.0.

FIGURE 13 is a diagram of a knockout construct  
5 carrying the 4.0 and 5.5kb SalI fragments from p $\alpha$ GT-S5.5 and p $\alpha$ GT-S4.0, which flank the Exon 9 SalI site.

FIGURE 14 depicts the 8.3kb and 6.4kb BglIII fragments that are diagnostic for the uninterrupted  $\alpha$ -1,3 galactosyltransferase gene and the targeted (inactivated)  
10  $\alpha$ -1,3 galactosyltransferase gene, respectively, using the probes identified in the text.

FIGURE 15 is a schematic representation of the generation of a knockout construct using the vector p $\alpha$ GT-S5.5 as the starting vector.

15 FIGURE 16 sets out the nucleotide sequence of a neomycin resistance cassette used in the construction of a DNA construct for interrupting the  $\alpha$ -1,3-GalT gene in mice.

FIGURE 17 is a diagram of one example of a final  
20 knockout construct that has been sequenced to confirm the identity, copy number and orientation of the various inserts.

FIGURE 18 is a Southern blot of genomic DNA from various murine ES cell lines transformed with the  
25 knockout construct of Figure 16, probed to reveal the diagnostic fragments depicted in Figure 14.

FIGURE 19 depicts the "long" PCR products derived from wild type and interrupted  $\alpha$ -1,3-GalT genes using the designated primers.

30 FIGURE 20 is a Southern blot of long PCR products obtained from wild type and knockout mice.

FIGURE 21 depicts the PCR products used for identification of the interrupted (targeted) galT locus.

FIGURE 22 shows PCR products generated from mice  
35 carrying interrupted (inactivated) GalT alleles.

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FIGURE 23 depicts the PCR products expected from PCR analysis of cDNA generated from  $\alpha$ -1,3-GalT mRNA in normal and knockout mice. The ferrochelataase primers and PCR fragment represent a control demonstrating that cDNA  
5 synthesis had occurred.

FIGURE 24 shows the PCR fragments generated from cDNA obtained from RNA isolated from kidney (K), heart (H) and liver (L) of a wild-type mouse (+/+), a mouse heterozygous for the interrupted  $\alpha$ -1,3-GalT allele (+/-)  
10 and a mouse homozygous for the interrupted  $\alpha$ -1,3-GalT allele (-/-).

FIGURE 25 is a graphical representation of the relative protection of spleen cells, derived from GalT knockout mice, from lysis by human serum.

15 FIGURE 26 is a representation of the nucleotide sequence and deduced amino acid sequence for murine T-LIF.

FIGURE 27 is a representation of the nucleotide sequence and deduced amino acid sequence for human T-LIF.

20 FIGURE 28 is a Western blot of LIF polypeptides expressed from transfected COS cells.

FIGURE 29 is a diagram of the expression plasmid used for transfection of the COS cells of Figure 27.

FIGURE 30 is a Southern blot of PCR-amplified cDNA  
25 from murine ES cells, using a LIF-specific probe.

#### DETAILED DESCRIPTION

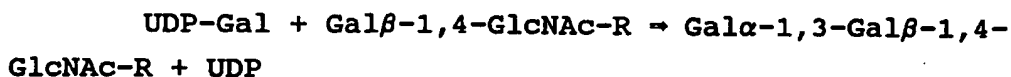
Evidence presented herein establishes that a substantial portion of human pre-formed, anti-pig xenoantibodies recognize a specific terminal galactose  
30 linkage on the surface of pig endothelial cells. As demonstrated in experiments carried out by the present inventors, it is possible to reduce the titers of preformed xenoantibodies by adsorption with immobilized antigens containing the appropriate epitopes. This leads



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to reduction or elimination of cellular responses associated with the hyperacute rejection response. Conversely, it is demonstrated to be possible to neutralize such antibodies by addition of appropriate carbohydrate antigens to human serum. In demonstrating the usefulness of these approaches, it was necessary to identify the relevant carbohydrate moieties and to demonstrate their efficacy in cultured cell systems and, importantly, in whole organs. As such, one approach to reducing or eliminating the hyperacute rejection response is identified as treatment of the recipient by eliminating or neutralizing the relevant antibody populations.

An alternative approach to xenotransplantation would be elimination of the relevant epitope(s) in the donor organ. This could be accomplished, for example, by reducing or eliminating expression of the gene(s) encoding the metabolic machinery responsible for formation of the epitopes. The epitope defined by the  $\alpha$ -1,3 galactose linkage (termed the GAL epitope) is generated by the enzyme UDP-galactose: $\beta$ -D-galactosyl-1,4-N-acetyl-D-glucosaminide  $\alpha$ -1,3 galactosyl-transferase (" $\alpha$ -1,3 galactosyltransferase" or " $\alpha$ -1,3-GalT"). This enzyme transfers galactose to the terminal galactose residue of N-acetylglucosamine-type carbohydrate chains and lactosaminoglycans. The reaction catalyzed by  $\alpha$ -1,3-GalT may be summarized as follows:



The  $\alpha$ -1,3-Gal T enzyme is found in most mammals, but is not present in Old World monkeys and humans. For purposes of xenotransplantation, it is significant that humans and Old World monkeys have naturally occurring xenoantibodies directed against the GAL epitope. The use of pig organs lacking the GAL epitope could reduce or

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eliminate the hyperacute rejection of such organs by human recipients. The utility of such an approach is buttressed by the present inventors' demonstration that the GAL epitope is, in fact, central to the hyperacute rejection phenomenon in cells and whole organs. One approach to obtaining such organs would be to generate pigs in which the gene encoding the  $\alpha$ -1,3-GalT enzyme is "knocked out" by homologous recombination.

#### Role of the GAL Epitope in Hyperacute Rejection

The present inventors have affinity purified antibodies directed against the GAL epitope (anti-GAL antibodies) from human serum. This was accomplished with affinity columns comprising the appropriate epitopes (e.g., galactosyl-galactose or melibiose) attached to a solid phase. Total anti-GAL IgG and IgM were obtained in one set of experiments. In an alternative approach, anti-GAL IgG was obtained by passage of serum over an affinity column with specificity for all proteins except albumin and IgG. The wash-through from this column was then applied to a galactosyl-galactose affinity column and purified anti-GAL IgG was collected as the eluate. The obtained anti-GAL IgG can be further purified by passage over a protein G column, which specifically binds IgG but not other antibody isotypes. Conversely, the wash-through from the above-described columns can be used as sources of total anti-GAL (IgG + IgM)-depleted serum or of anti-GAL IgG-depleted serum in further experiments. Preferably, the anti-GAL antibody preparations are characterized for protein content, molecular weight and purity, and for antibody class and isotype.

To demonstrate the role of the GAL epitope in the hyperacute rejection response, it is necessary, first, to establish that IgG and IgM anti-GAL antibodies react with porcine cells and tissues. The present inventors

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investigated the binding of human anti-GAL antibodies to porcine cells and tissues using immunofluorescent staining. In this technique, selected human antibody preparations are reacted with intact porcine cells and  
5 then reacted with signal antibody comprising non-human anti-human IgG or IgM labeled with fluorescein isothiocyanate (FITC). Stained cells may be detected and quantified with a fluorescence-activated cell sorter (FACS) or other appropriate detection means. Other  
10 methods for detecting the presence of a bound antibody on a cell surface, for example through use of enzyme-labeled signal antibody reagents, are known to the skilled artisan.

Total anti-GAL (IgM and IgG), as well as  
15 purified anti-GAL IgG, stained cells from a porcine epithelial cell line (PK<sub>1</sub>) as well as cells from a porcine aortic endothelial cell line (PAE). Neither anti-GAL (total IgM + IgG) antibody-depleted serum nor anti-GAL IgG-depleted serum gave detectable staining. To  
20 further investigate the specificity of the response, it is desirable to determine whether or not reactivity of the antibodies with porcine cells can be diminished or eliminated by prior exposure to one or more molecules suspected of comprising the epitope(s) in question. In  
25 this regard, the present inventors have established that antibody binding is inhibited by galactose and by disaccharides having terminal galactose residues in the  $\alpha 1$  configuration. Staining was not inhibited with sugars having a terminal galactose in a  $\beta 1 \rightarrow 4$  configuration.  
30 These results demonstrate the specificity of the antibody binding and the ability of appropriate sugars to inhibit such binding.

Reactivity of anti-GAL antibodies with cultured pig cells was confirmed using tissue sections of pig  
35 organs. Again, using a fluorescent signal antibody

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system, staining was seen with total anti-GAL IgM + IgG and with purified anti-GAL IgG but not with the anti-GAL antibody-depleted sera. Staining was particularly strong with kidney, heart and liver endothelium, with heart  
5 endocardium and with bile duct epithelium. The tissue binding was inhibited with melibiose but was not inhibited by other disaccharides not representative of the GAL epitope.

These data clearly indicate that the GAL epitope  
10 is expressed at high levels on the endothelial cells of arteries, veins and capillaries of porcine kidney, heart and liver. In a xenograft situation, the endothelial cells of these vessels come into direct contact with the anti-GAL antibodies in human serum. The above results  
15 are consistent with evidence that binding of these antibodies (with attendant complement activation) is a key component of the hyperacute rejection response.

To further investigate the specificities of naturally occurring xenoantibodies in human serum  
20 directed against porcine antigens, the ability of human serum to cause agglutination of pig red blood cells was investigated. These studies revealed the presence of high levels of such antibodies in human serum. Moreover, sugars such as melibiose, stachyose, galactose and  
25 fucose, having terminal residues in the  $\alpha 1-6$  configuration, were found to inhibit agglutination in the  $\mu\text{M}$  to  $\text{mM}$  range. Sugars with other configurations were only inhibitory at very high doses, where the observed effects are likely due to simple changes in osmolarity or  
30 other non-specific mechanisms.

The above investigations establish a potential role for naturally occurring, human anti-GAL xenoantibodies in the complement-mediated destruction underlying hyperacute rejection. However, it is  
35 preferable to directly examine complement-mediated

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destruction of porcine cells in order to confirm the specificity of the GAL epitope and of anti-GAL antibodies in the process of lysis. To this end, the present inventors have examined the ability of human serum to  
5 cause lysis of porcine cells.

To investigate complement-mediated destruction of cells, it is necessary to employ one or more assays that provide quantitative data on cell lysis. Preferably, such assays measure a cell-sequestered component that is  
10 released into the medium upon complement-mediated cell lysis. Such experiments should control for involvement of complement in the induced lysis by employing both native complement proteins as well as heat-inactivated complement. The present inventors have used a  $^{51}\text{Cr}$ -  
15 release assay and a lactate dehydrogenase (LDH)-release assay to investigate the complement-mediated lysis of porcine epithelial and endothelial cells by human serum.

In the  $^{51}\text{Cr}$ -release assay, porcine cells were pre-labeled with  $^{51}\text{Cr}$  and then incubated in the presence  
20 of heat-inactivated human serum plus rabbit complement (PAE's) or human complement in non-heat-inactivated normal human serum (PK<sub>1</sub>'s). Release of  $^{51}\text{Cr}$  into the medium was measured with a gamma counter following addition of scintillation fluid. In the LDH-release  
25 assay, cells were labeled with LDH as per the manufacturer's instructions (Promega, USA). Release of LDH into the medium was measured using an ELISA format, with absorbance read at 492nm. For both assays, the ability of various sugars to inhibit the complement-  
30 induced lysis was also tested.

Similar results were obtained with the two unrelated porcine cell lines, PAE and PK<sub>1</sub>, using both types of assays. The results clearly demonstrate that naturally occurring xenoantibodies (NXAb's) are  
35 responsible for initiating the complement-induced lysis

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of porcine cells. The present inventors have also established that IgM and not IgG antibodies are responsible for the lysis in this system. Moreover, heat inactivation of the complement preparations prevented  
5 lysis, providing further evidence that lysis of the porcine cells is a complement-dependent phenomenon. The present inventors have also shown that melibiose, but not lactose, protects the porcine cells from lysis. Importantly, the concentrations of sugar found to be  
10 effective in these studies covered the physiological range of blood sugar, i.e., about 10mM.

These results indicate that the anti-GAL NXAb's in normal human serum are primarily responsible for lysis of the porcine cells. As such, the binding of anti-GAL  
15 NXAb's to the endothelial cells lining the blood vessels of a porcine xenograft, with attendant activation of the complement cascade, is likely to be a key component of the hyperacute rejection of porcine xenografts. This would also be the case with organs from other discordant  
20 species, such as rodents, sheep, cows and goats, all of which have active  $\alpha$ -1,3-GalT genes in their genomes.

These conclusions are further supported in a whole-organ study performed by the present inventors. For this study, isolated and perfused rat hearts were  
25 used to further demonstrate the involvement of anti-GAL xenoantibodies in hyperacute rejection. Rat hearts were connected to a Langendorff perfusion apparatus, as described in Doring and Dehnert, The Isolated Perfused Heart According to Langendorff, Bionestechnik-Verlag  
30 March GmbH, D7806, West Germany. The connected hearts were then stabilized by perfusion with a physiological buffer system, and perfused with the same buffer containing either melibiose or lactose (10mM). Human plasma was then added to a final concentration of 13% and

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the effect of the added sugar on heart rate, strength of contraction and output were measured.

These results demonstrate in a whole-organ system that:

5           1)    Perfusion with unmodified human plasma causes rapid loss of function.

          2)    Perfusion of a rat heart with human plasma in the presence of melibiose, which competes for binding with the anti-GAL antibodies, prolongs heart survival and  
10 output. Lactose, however, which does not compete for binding with the anti-GAL antibodies, does not prolong heart survival.

          3)    Perfusion of a rat heart with anti-GAL antibody-depleted plasma prolongs heart survival and  
15 output.

          4)    If purified anti-GAL antibodies are added back to anti-GAL antibody-depleted plasma, the heart rapidly loses function

The present inventors' experiments with cultured  
20 cells, tissues and whole organs provide important confirmation that anti-GAL antibodies are a critical element in the hyperacute rejection response. Moreover, the disclosed results point to various approaches that can be employed to eliminate or reduce the hyperacute  
25 rejection of xenogeneic mammalian organs by humans.

For example, the intravenous administration of the specific disaccharide galactose  $\alpha$  1-3 galactose will block the naturally occurring anti-GAL antibodies of all classes and prevent them binding to their specific  
30 epitopes on the surface of the endothelial cells of the xenograft, thus preventing them from initiating or participating in hyperacute rejection. The present inventors' results indicate that the concentration of galactose  $\alpha$  1-3 galactose required to achieve this effect

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is in a physiologically tolerated range. The experiments also indicate that various other carbohydrates can be substituted for the specific disaccharide. These include the monosaccharide galactose and various other di-, tri-  
5 or tetra-saccharides in which there is a terminal  $\alpha$  galactose linked to the next sugar via position 1. These other sugars include, but are not limited to, melibiose and stachyose.

Likewise, prior to xenotransplantation, all or a  
10 substantial portion of total IgM (that is, IgM of all specificities) can be removed from the serum of the patient by extracorporeal immunoabsorption. Alternatively, anti-GAL antibodies of all classes can be removed by extracorporeal immunoabsorption. Most  
15 preferably, the patient's pre-formed natural anti-GAL IgM antibodies can be removed. In this way, many or most of the primary immunological agents of the hyperacute response are eliminated, resulting in reduction or elimination of the response following  
20 xenotransplantation.

#### The $\alpha$ -1,3-GalT Gene as a Target for Suppressing the GAL Epitope

The present inventors have succeeded in cloning the entire coding region of the porcine  $\alpha$ -1,3-GalT gene.  
25 This is desirable for full exploitation of the gene in genetic engineering of pigs for purposes of human xenotransplantation. Previous attempts to obtain the entire coding region of the porcine gene have, to the knowledge of the inventors, failed to generate the 5'  
30 coding regions. See, e.g., Dabkowski et al., Transplant. Proc. 25: 2921 (1993). The present inventors have employed a PCR-based approach to generate the full sequence. In designing the primers and experimental conditions required to obtain the 5' and 3' regions of



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the gene, the present inventors overcame significant theoretical and practical obstacles to success.

Primers were selected on the basis of careful analysis of published sequences for the murine, bovine  
5 and human  $\alpha$ -1,3-GalT genes, the only published sequences available for this purpose. The present inventors' analysis revealed that in the reported sequence of the bovine cDNA, exon 3 (which is in the 5'-untranslated region) is missing. This had not been reported in the  
10 literature. Thus, in order to find appropriate regions for deriving useful primer sequences, the mouse and bovine sequences had to be realigned. Even with the appropriate realignment, however, only one island of about 20 base pairs (bp) in the 5' untranslated region  
15 displayed the desired homology (19 out of 20 bp) for design of a PCR oligonucleotide. The fact that the 5' untranslated regions of the mouse and bovine genes do not seem substantially related even upon optimal alignment would not be considered unusual by the ordinary skilled  
20 artisan. This is because the 5' untranslated regions are often not well conserved between species. As such, the natural inclination would be to perform a less-than-exhaustive analysis and to conclude that design of PCR oligonucleotides based on homology from this region was  
25 unlikely to be successful.

In the downstream 3'-untranslated region, the homology is less than obvious again. Various insertions and deletions had to be made in order to obtain proper alignment of the mouse and bovine sequences. Moreover,  
30 to obtain a region of appropriate homology for design of PCR oligonucleotides, it was necessary to select a region approximately 200 bp downstream of the stop codon. Finally, to get the 5' and 3' primers to work properly, the present inventors found it necessary to drop the  
35 annealing temperature by 9°C. These technical and

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theoretical hurdles to successful use of a PCR-based approach were overcome by the present inventors and allowed the entire coding sequence to be determined.

Analysis of the nucleotide sequence indicates that  
5 a counterpart to murine exon 3 in the 5' untranslated region is not found in the porcine gene. The porcine sequence is similar to the bovine sequence in this regard. Analysis of the amino acid sequence demonstrates that the structure of the porcine  $\alpha$ -1,3-GalT is similar  
10 to that of other glycosyltransferases, and in particular is closely related to bovine and murine  $\alpha$ -1,3-GalTs. In each of these enzymes a short cytoplasmic amino-terminal domain of about 6 residues precedes a hydrophobic membrane-anchoring domain (extending from residues 7 to  
15 22). The stem region, which serves as a flexible tether, and the catalytic domain, which catalyses the synthesis of  $\alpha$ -1,3-GAL linkages, are located in the lumen of the Golgi and extend from amino acid 23 to the carboxyl terminus at amino acid 371. The precise boundary between  
20 the stem and catalytic domains is not well-defined. Based on the suggested characteristics of the stem region, it appears to be the least conserved region and is rich in glycine and proline residues. Paulson and Colley, J. Biol. Chem. 264: 17615 (1989); Joziassse et  
25 al., J. Biol. Chem. 267: 5534 (1992). The stem/catalytic boundary may occur around amino acid 60.

To generate constructs for inactivating genes by homologous recombination, the gene is preferably interrupted within an appropriate coding exon by  
30 insertion of an additional DNA fragment. Upon analysis of the full-length porcine nucleic acid sequence, the present inventors have identified exons 4, 7, 8 and 9 as preferred locations for disruption of the gene by homologous recombination. However, identification of  
35 these exons as preferred sites should not be construed as

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limiting the scope of the present invention, as interruptions in exons 5 and 6 may be useful in particular cell types or in situations where less-than-complete inhibition of  $\alpha$ -1,3-GalT gene expression is  
5 desired. Moreover, regulatory elements associated with the coding sequence may also present useful targets for inactivation.

In a preferred embodiment, a Sall site located within exon 9 of the mouse  $\alpha$ -1,3-GalT gene at codons 221-  
10 222 is chosen as the site for disruption of the murine coding sequence. For disruption of the porcine sequence, it is noted that the amino acids encoded by the corresponding porcine nucleotides are conserved, although the Sall site is not. In a preferred embodiment for  
15 inactivation of the porcine gene, a Sall site is engineered into the corresponding location of the pig sequence for convenient construction of a knockout sequence. Sall cuts only rarely in genomic DNA. Since multiple restriction sites can be a problem in  
20 manipulating large fragments of DNA, the presence of a Sall site in the exon is very useful since it is not likely that other Sall sites will be present at other locations in the knockout constructs.

A gene coding for a selectable marker is generally  
25 used to interrupt the targeted exon site by homologous recombination. Preferably, the selectable marker is flanked by sequences homologous to the sequences flanking the desired insertion site. Thomas and Capecchi, Cell 51: 503-12 (1987); Capecchi, Trends in Genetics 5: 70-76  
30 (1989). It is not necessary for the flanking sequences to be immediately adjacent to the desired insertion site. The gene imparting resistance to the antibiotic G418 (a neomycin derivative) frequently is used, although other antibiotic resistance markers (e.g., hygromycin) also may  
35 be employed. Other selection systems include negative-

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selection markers such as the thymidine kinase (TK) gene from herpes simplex. Any selectable marker suitable for inclusion in a knockout vector is within the scope of the present invention.

5           However, it is possible that in some circumstances it will not be desirable to have an expressed antibiotic resistance gene incorporated into the cells of a transplanted organ. Therefore, in a preferred embodiment, one or more genetic elements are included in  
10 the knockout construct that permit the antibiotic resistance gene to be excised once the construct has undergone homologous recombination with the  $\alpha$ -1,3-GalT gene.

          The FLP/FRT recombinase system from yeast  
15 represents one such set of genetic elements. O'Gorman et al., Science 251, 1351-1355 (1991). FLP recombinase is a protein of approximately 45 kD molecular weight. It is encoded by the FLP gene of the 2 micron plasmid of the yeast *Saccharomyces cerevisiae*. The protein acts by  
20 binding to the FLP Recombinase Target site, or FRT; the core region of the FRT is a DNA sequence of approximately 34 bp. FLP can mediate several kinds of recombination reactions including excision, insertion and inversion, depending on the relative orientations of flanking FRT  
25 sites. If a region of DNA is flanked by direct repeats of the FRT, FLP will act to excise the intervening DNA, leaving only a single FRT. FLP has been shown to function in a wide range of systems, including in the cultured mammalian cell lines CV-1 and F9, O'Gorman et  
30 al., Science 251: 1351 (1991), and in mouse ES cells, Jung et al., Science 259: 984 (1993).

          Targeted cells carrying a genomic copy of an antibiotic resistance gene flanked by direct repeats of the FRT are supplied with FLP recombinase by 1)  
35 introduction into cells of partially purified FLP protein

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by electroporation, or 2) transfection with expression plasmids containing the FLP gene. In this way, the antibiotic resistance gene is deleted by action of the FLP recombinase, and cells are generated that contain the  
5 inactivated  $\alpha$ -1,3-GalT gene and are free of the exogenous antibiotic resistance gene.

Due to the relative infrequency of homologous recombination in targeted cells, most such cells will carry only one inactivated allele of the target gene.  
10 That is, the great majority of cells taken through a single round of transformation with an appropriate knockout construct will be heterozygotes. As used herein, the term "transformed" is defined as introduction of exogenous DNA into the target cell by any means known  
15 to the skilled artisan. These methods of introduction can include, without limitation, transfection, microinjection, infection (with, for example, retroviral-based vectors), electroporation and microballistics. The term "transformed," unless otherwise indicated, is not  
20 intended herein to indicate alterations in cell behavior and growth patterns accompanying immortalization, density-independent growth, malignant transformation or similar acquired states in culture.

Although heterozygous cells can be used in the  
25 methods of the present invention, various manipulations can be employed to generate homozygous cells in culture. For example, homozygous cells can be generated by performing a second homologous recombination procedure on cells heterozygous for the inactivated allele. If the  
30 knockout construct used in the initial transformation carried the  $neo^R$  gene, a second construct may be employed in a second round of transformation in which the  $neo^R$  gene is replaced with a gene conferring resistance to a separate antibiotic (e.g., hygromycin). Cells resistant  
35 to both G418 and hygromycin can be screened by Southern

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blots in order to detect any "double knockouts" (i.e., homozygotes). Both antibiotic resistance genes can be removed subsequently in a single procedure using FLP recombinase. By maintaining selection with G418, this approach ensures that the second construct does not simply replace the previously knocked-out allele, leaving the cells heterozygous.

Alternatively, the  $\text{neo}^R$  gene can be deleted from an original heterozygous cell using FLP recombinase and a second knockout procedure conducted using the original  $\text{neo}^R$  gene-containing construct. Double knockouts could be detected by Southern analysis. The newly introduced  $\text{neo}^R$  gene then could be deleted by FLP recombinase. This alternative approach does not allow one to direct the knockout construct specifically to the non-inactivated allele. Nevertheless, screening of appropriate numbers of targeted cells can lead to identification of cells homozygous for the inactivated locus.

#### Cellular Vehicles for Incorporation of Knockout Constructs

To create animals having a particular gene inactivated in all cells, it is necessary to introduce a knockout construct into the germ cells (sperm or eggs, i.e., the "germ line") of the desired species. Genes or other DNA sequences can be introduced into the pronuclei of fertilized eggs by microinjection. Following pronuclear fusion, the developing embryo may carry the introduced gene in all its somatic and germ cells since the zygote is the mitotic progenitor of all cells in the embryo. Since targeted insertion of a knockout construct is a relatively rare event, it is desirable to generate and screen a large number of animals when employing such an approach. Because of this, it can be advantageous to work with the large cell populations and selection criteria that are characteristic of cultured cell

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systems. However, for production of knockout animals from an initial population of cultured cells, it is necessary that a cultured cell containing the desired knockout construct be capable of generating a whole  
5 animal. This is generally accomplished by placing the cell into a developing embryo environment of some sort.

Cells capable of giving rise to at least several differentiated cell types are hereinafter termed "pluripotent" cells. Pluripotent cells capable of giving  
10 rise to all cell types of an embryo, including germ cells, are hereinafter termed "totipotent" cells. Totipotent murine cell lines (embryonic stem, or "ES" cells) have been isolated by culture of cells derived from very young embryos (blastocysts). Such cells are  
15 capable, upon incorporation into an embryo, of differentiating into all cell types, including germ cells, and can be employed to generate animals lacking a functional  $\alpha$ -1,3-GalT gene. That is, cultured ES cells can be transformed with a knockout construct and cells  
20 selected in which the  $\alpha$ -1,3-GalT gene is inactivated through insertion of the construct within, for example, an appropriate exon. In fact, ES cell lines have been derived for both mice and pigs. See, e.g., Robertson, Embryo-Derived Stem Cell Lines. In: Teratocarcinomas and  
25 Embryonic Stem Cells: A Practical Approach (E.J. Robertson, ed.), IRL Press, Oxford (1987); PCT Publication No. WO/90/03432; PCT Publication No. 94/26884. Generally these cells lines must be propagated in a medium containing a differentiation-inhibiting  
30 factor (DIF) to prevent spontaneous differentiation and loss of mitotic capability. Leukemia Inhibitory Factor (LIF) is particularly useful as a DIF. Other DIF's useful for prevention of ES cell differentiation include, without limitation, Oncostatin M (Gearing and Bruce, The  
35 New Biologist 4: 61-65 (1992); personal communication

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from A. Smith), interleukin 6 (IL-6) with soluble IL-6 receptor (sIL-6R) (Taga et al., Cell 58: 573-81 (1989); personal communication from A. Smith), and ciliary neurotropic factor (CNTF) (Conover et al., Development 5 19: 559-65 (1993)). Other known cytokines may also function as appropriate DIF's, alone or in combination with other DIF's.

As a useful advance in maintenance of ES cells in an undifferentiated state, the present inventors have 10 identified a novel variant of LIF. In contrast to the previously identified forms of LIF which are extracellular, this new form of LIF (hereinafter T-LIF) is intracellularly localized. The transcript was cloned from murine ES cells using the RACE technique, Frohman et 15 al., Proc. Natl. Acad. Sci. USA 85: 8998-9002 (1988), and subjected to sequence analysis. Analysis of the obtained nucleic acid sequence and deduced amino acid sequence indicates that T-LIF is a truncated form of the LIF sequence previously reported in the literature.

20 Expression of the T-LIF nucleic acid in an appropriate host cell yields a 17 kD protein that is unglycosylated. This protein is useful for inhibiting differentiation of murine ES cells in culture. The protein is expected to have a similar activity with porcine cells, since murine 25 D-LIF is effective at inhibiting both murine and porcine ES cell differentiation. The present inventors have also determined the sequence of the human form of T-LIF.

To generate a knockout animal, ES cells having at least one inactivated  $\alpha$ -1,3-GalT allele are identified 30 and incorporated into a developing embryo. This can be accomplished through injection into the blastocyst cavity of a murine blastocyst-stage embryo, by injection into a morula-stage embryo, by co-culture of ES cells with a morula-stage embryo, or through fusion of the ES cell 35 with an enucleated zygote. The resulting embryo is



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raised to sexual maturity and bred in order to obtain animals, all of whose cells (including germ cells) carry the inactivated  $\alpha$ -1,3-GalT allele. If the original ES cell was heterozygous for the inactivated  $\alpha$ -1,3-GalT allele, several of these animals must be bred with each other in order to generate animals homozygous for the inactivated allele.

Although direct microinjection of DNA into eggs does not generate the large numbers of recombination events obtained through transfecting large numbers of cultured cells, nevertheless direct injection of eggs can be a useful approach since this avoids the special manipulations (see above) required to turn a cultured cell into an animal. This is because fertilized eggs are, of course, quintessentially "totipotent" - i.e., capable of developing into an adult without further substantive manipulation other than implantation into a surrogate mother. To enhance the probability of homologous recombination when eggs are directly injected with knockout constructs, it is useful to incorporate at least about 8 kb of homologous DNA into the targeting construct. In addition, it is also useful to prepare the knockout constructs from isogenic DNA. For example, for injection of porcine eggs, it is useful to prepare the constructs from DNA isolated from the boar whose sperm are employed to fertilize the eggs used for injection.

Embryos derived from microinjected eggs can be screened for homologous recombination events in several ways. For example, if the GalT gene is interrupted by a coding region that produces a detectable (e.g., fluorescent) gene product, then the injected eggs are cultured to the blastocyst stage and analyzed for presence of the indicator polypeptide. Embryos with fluorescing cells, for example, are then implanted into a

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surrogate mother and allowed to develop to term. Alternatively, injected eggs are allowed to develop and the resulting piglets analyzed by polymerase chain reaction (PCR) or reverse transcription PCR (RT/PCR) for  
5 evidence of homologous recombination.

#### Characterization of Knockout Animals

Animals having either one (heterozygous) or two (homozygous) inactivated GALT genes are characterized to confirm the expected alterations in gene expression and  
10 phenotypic effect. For example, GALT mRNA should be absent from homozygous knockout animals. This can be confirmed, for example, with reverse transcription PCR (RT-PCR) using appropriate GALT-specific primers. In addition, various tests can be performed to evaluate  
15 expression of the GAL epitope in homozygous knockout animals. For example, anti-GAL antibodies and IB<sub>4</sub> Lectin (which has an exclusive affinity for terminal  $\alpha$ -D-galactosyl residues) can be used in various assay or immunohistological formats to test for the presence of  
20 the GAL epitope in an array of tissues. As another indication of GAL epitope status, lysis of cells by human serum can be tested through use of a <sup>51</sup>chromium release assay.

#### EXAMPLE 1

##### 25 Affinity Purification of Human Anti-GAL Antibodies

Anti-GAL antibodies were purified from normal heat inactivated AB serum (from CS1, Parkville, Victoria, Australia) using the following sets of procedures.

##### A. Preparation of total anti-GAL (IgG+IgM) antibodies

30 The following procedures are performed at 4°C.

1. Desalt 15-30ml serum (in 3ml batches) by passage through a pre-equilibrated (20ml application buffer: 20mM K<sub>2</sub>HPO<sub>4</sub>, 30mM NaCl, pH 8) Econo Pac 10DG (Bio-Rad, Richmond, USA) column. Alternatively, for large scale

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preparations, desalt by dialysis exhaustively against application buffer.

2. Wash column with 4ml aliquots of application buffer. Collect and pool column eluates.

5 3. Apply pooled desalted serum to a pre-equilibrated (20ml application buffer) Synsorb 115 (galactosyl-galactose; Chembiomed, Alberta, Canada) or D(+) Melibiose-Agarose (Sigma) affinity column (5ml-50 ml depending on the yield required).

10 4. Collect run-through (partially anti-GAL-depleted) and reapply to column. Repeat process 3 times to ensure complete removal of anti-GAL antibodies. The wash-through from the 3rd passage through the Synsorb column is collected and the volume adjusted to the original  
15 volume of the serum with phosphate-buffered saline (PBS) pH 7 +0.05% azide. This is used as a source of anti-GAL antibody-depleted serum.

5. Wash column with PBS pH 8 until the eluate is protein free (O.D. 280nm=0).

20 6. Elute anti-GAL antibodies with 3.5M KSCN, pH 7.5. Collect 4ml fractions, determine the O.D. 280 and pool peak fractions (usually 1-6).

7. Concentrate anti-GAL antibodies using CF25 ultrafiltration cones (Amicon, Danvers, USA). Add 7ml of  
25 the pooled fractions containing anti-GAL antibodies to spin cone and centrifuge (2,000 RPM, 10min, 4°C). Refill cone and recentrifuge until volume is reduced to 3-5ml.

8. To dilute the KSCN, adjust vol. to 7ml with PBS and centrifuge (2,000 RPM, 10min, 4°C). Repeat process a  
30 further 10 times.

9. Remove sample containing anti-GAL antibodies from cone using plastic pipette; rinse cone with PBS pH7 +0.05% azide.

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B. Preparation of IgG anti-GAL antibodies

The following procedures are performed at 4°C.

1. Desalt 15-30 ml serum (in 3ml batches) by passage through a pre-equilibrated (20ml application buffer) Econo Pac 10DG (Bio-Rad, Richmond, USA) column. Alternatively for large scale preparations desalt by dialysis exhaustively against application buffer.
2. Wash column with 4ml aliquots of application buffer. Collect and pool column eluates.
- 10 3. Apply desalted serum to a pre-equilibrated (30ml application buffer) Affi-Blue column (Bio-Rad, Richmond, USA) (Affi-Blue binds all proteins except albumin and IgG).
4. Wash column with 20ml application buffer to elute 15 IgG enriched fraction.
5. Apply IgG enriched fraction to a pre-equilibrated (20ml application buffer, pH 8.0) Synsorb 115 (galactosyl-galactose; Chembiomed, Alberta, Canada) affinity column (5ml).
- 20 6. Collect run-through and reapply to column. Repeat process 3 times to ensure complete removal of anti-GAL antibodies. The wash-through from the 3rd passage through the Synsorb column is collected and the volume adjusted to the original volume of the serum with PBS pH 25 7 +0.05% azide. This is used as a source of control anti-GAL-depleted IgG.

In some cases anti-GAL IgG was further purified using a protein G column, which efficiently binds IgG but not other antibody isotypes. IgG was then eluted from 30 the protein G column using glycine pH 2.4.

All anti-GAL antibody preparations were analyzed for the following:

- a. Protein content was determined using the Bradford colorimetric 35 method (Bradford, M.M 1976,

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Anal. Biochem. 72:248-254),  
using purified human IgG as the  
standard.

- 5           b. Molecular weight and purity were  
determined using polyacrylamide  
gel electrophoresis according to  
method described by Laemli,  
Nature (London) 227: 680 (1970),  
and protein was detected in the  
10           gels by silver staining using  
standard kit reagents (Amersham,  
UK).
- c. Antibody class and isotype were  
determined by radial  
15           immunodiffusion using standard  
techniques as set out in Rose et  
al. (eds.), Manual of Clinical  
Laboratory Immunology, American  
Society for Microbiology,  
20           Washington, D.C. IgG anti-GAL  
preparations were found to  
contain all subclasses, with  
IgG2 predominating.

25

## EXAMPLE 2

Reactivity of IgG and IgM Anti-GAL Antibodies and  
Depleted Serum with Porcine Cells and Tissues

## I. CELLS

Reactivity of IgG and IgM anti-GAL antibodies was  
30 assessed using either porcine aortic endothelial cells  
(prepared by the inventors as described below) or porcine  
epithelial cell line LLC PK<sub>1</sub> (PK<sub>1</sub>), obtained from the

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American Type Culture Collection (ATCC), Accession No. CRL1392.

A. Isolation and culture of porcine aortic endothelial cells (PAE's)

- 5 Pigs were blood typed (using human typing reagents) to identify "O-type" pigs, i.e, pigs unreactive with antibodies to A or B human red blood cell antigens. Aortas were excised from "O-type" pigs, then transported from the abattoir to the laboratory on ice. PAE's were  
10 isolated by collagenase treatment as described by Gimbrone et al., J. Cell Biol. 60: 673-84 (1974). PAE's were cultured in RPMI medium containing 10% fetal calf serum (FCS), supplemented with 150µg/ml endothelial cell supplement (Sigma) and 50µg/ml heparin (Sigma). The  
15 cells were identified as endothelial cells by their typical cobblestone morphology and by their immunoreactivity with Factor VIII antibodies, as identified using immunofluorescence. In all the assays described below, the PAE's were used between the 8th and  
20 12th passages.

B. Tissue Culture: Maintenance of PK-1 and PAE cell lines

- All tissue culture was performed in a laminar flow hood, using appropriate tissue culture sterile technique.  
25 All tissue culture reagents, unless otherwise indicated, were purchased from CSL, Melbourne, Australia. Media were constituted as follows:

PK-1 Culture Medium:

- |    |  |                                     |
|----|--|-------------------------------------|
| 30 | DMEM (Cytosystems, Castle Hill, Australia) | 500ml                               |
|    | FCS (CSL, Melbourne, Australia)            | 37.5ml                              |
|    | Glutamine (200mM) (Cytosystems)            | 5ml                                 |
|    | Hepes (1M) (CSL)                           | 7.5ml                               |
|    | Penicillin (CSL)                           | 0.5ml (10 <sup>5</sup> U/ml final)  |
|    | Streptomycin (CSL)                         | 0.5ml (10 <sup>5</sup> µg/ml final) |

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## PAE - Culture Medium:

	RPMI (CSL)	90ml
	FCS (CSL)	10ml
	Endothelial cell	
5	supplement (3mg/ml) (Sigma)	1.5ml
	Heparin (10mg/ml) (CSL)	0.5ml

Endothelial cell supplement was purchased from Sigma Chem. Co. (St. Louis, Missouri, USA) as a lyophilized powder, resuspended in sterile HBBS, and 3ml aliquots stored at 4°C.

Heparin (Sigma, Missouri, USA) (10mg/ml) - dissolved in PBS  
(0.22µm) - filter sterilized  
15 Hanks Buffer - purchased from Cytosystems

The following general procedures were used in propagating the cell lines.

- 1) Pour off old medium
- 20 2) Rinse cells twice with sterile PBS
- 3) Add 3ml of TED (0.05 M trypsin, 0.53 M EDTA, Gibco, NY, USA)
- 4) Incubate 10 min. in CO<sub>2</sub> incubator at 37°C
- 5) Add 7ml RPMI with 10% FCS
- 25 6) Resuspend cells and transfer to a sterile 10ml tube
- 7) Centrifuge for 5min at 1200 rpm, discard supernatant
- 8) Resuspend cells in RPMI with 10% Newborn Bovine Serum (NBS) and repeat centrifugation
- 30 9) Resuspend cells in 1ml DMEM (PK-1's) or RPMI (PAE's) (with additives, as described above).
- 10) Add 10ml medium and the appropriate volume of cell suspension to achieve the desired dilution for each 75cm<sup>2</sup> tissue culture
- 35

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flask, and return to humidified CO<sub>2</sub> incubator.

C. Antibody staining and FACS analysis

- 5           1) Add 2ml TED to a 75cm<sup>2</sup> culture flask containing PK-1 or PAE's, and incubate at room temperature for 10 min.
- 2) Add RPMI plus 10% FCS (5ml) to neutralize trypsin.
- 10          3) Pellet cells by centrifugation (700g, 5 min, 4°C).
- 4) Wash cells by resuspension and centrifugation in Hanks Buffer (x2).
- 5) Pellet cells by centrifugation (700g, 5 min, 4°C).
- 15          6) Resuspend cell pellet in Hanks buffer containing purified anti-GAL antibodies, GAL-depleted serum or GAL-depleted IgG and incubate at 4°C for 60 min. All antibodies were used undiluted, or diluted 1:2 or 1:4 in Hanks buffer.
- 20          7) Add 1ml of Hanks Buffer, pellet cells by centrifugation and aspirate off supernatant.
- 25          8) Resuspend pellet in FITC-labelled sheep-anti-human IgG Fab2 or IgM Fab2 (Silenus, Hawthorn, Australia) diluted 1:80 in Hanks buffer.
- 30          9) Incubate for 30 min. at 4°C.
- 10) Wash three times with Hanks buffer; resuspend pellet from final wash in 0.5ml Hanks buffer.



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- 11) Analyze stained samples using a FACScan II (Becton Dickinson) according to the manufacturer's instructions.

The specificity of the anti-GAL antibody binding to porcine cells was determined by examining the ability of sugars of various structures to inhibit antibody binding. In these competition studies the anti-GAL antibodies were pre-incubated with sugar (0.1M) at 37°C for 30 min before adding to the cells.

10 D. Results

Using immunofluorescence it was found that total anti-GAL (IgM & IgG) and purified anti-GAL IgG stained both PK-1 and PAE's cells. On the other hand, neither the total anti-GAL antibody-depleted serum nor the anti-GAL IgG-depleted serum gave detectable staining over background. The staining with anti-GAL IgM and/or IgG was inhibited with purified galactose and with disaccharides having terminal galactose residues in the  $\alpha$ 1-configuration such as melibiose (6-O- $\alpha$ -D-galactopyranosyl- D-glucose) and stachyose ( $\alpha$ -D-Gal-[1->6]- $\alpha$ -D-Glc-[1->2]- $\beta$ -D-Fru). Staining was not inhibited with sugars such as lactose (4-O- $\beta$ -D-galactopyranosyl- $\alpha$ -D-glucose), which has a terminal galactose residue, but in a  $\beta$ 1->4 configuration. The results of one such experiment are represented in Figure 1. PAE's were stained with anti-GAL antibody alone (GAL:PBS) or with anti-GAL antibody that had been pre-incubated with either melibiose (GAL:MELIBIOSE), galactose (GAL:GALACTOSE) or lactose (GAL:LACTOSE). Anti-GAL antibody staining was approximately 10 fold less in the samples containing melibiose and galactose, but was not affected significantly by lactose.

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## II. TISSUES

A. Methods

Pig kidney was fixed in formalin and dehydrated before embedding in Paraplast. Pig heart and liver were  
5 fixed in paraformaldehyde-lysine-periodate fixative and snap frozen in O.C.T. embedding compound (10.24% w/w polyvinyl alcohol, 4.26% w/w polyethylene glycol, 85.50% w/w nonreactive ingredients; Tissue Tek®, Miles, Inc., Elkhart, Indiana, USA). Four  $\mu$ m-thick sections of pig  
10 heart and liver and 2  $\mu$ m-thick sections of kidney were incubated with purified anti-GAL antibodies (undiluted, 1:2 and 1:4) for 60 min. and then incubated with a fluorescein isothiocyanate (FITC)-conjugated sheep anti-human immunoglobulin F(ab') fragment (Silenus  
15 Laboratories, Hawthorn, Australia) (1:100) for 30 min. or a peroxidase-conjugated rabbit anti-human IgG (Dakopatts, Glostrup, Denmark) (1:50) for 60 min. Control sections were analyzed for autofluorescence, with the secondary antibody alone, or with the anti-GAL-depleted IgG or  
20 normal pig serum as the primary antibody. No staining was detected. The specificity of the anti-GAL antibodies was tested by pre-incubating sections of pig renal cortex with a variety of sugars, including melibiose, lactose, sucrose and glucose at 0.1M.

25 B. Results

As with the analyses performed on the pig cells using immunofluorescence, total anti-GAL IgM + IgG, purified anti-GAL IgG, but not the anti-GAL IgM and/or IgG-depleted sera, stained all pig tissues examined. The  
30 individual staining parameters varied from organ to organ as set out below:

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Immunostaining of Pig Tissues with Anti-GAL Antibodies:

	<u>Tissue</u>	<u>Anti-GAL Reactivity</u>	<u>Staining Intensity</u>
5	Kidney	Proximal and distal convoluted tubules	Variable
		Endothelium: Intertubular sinusoids	Variable
		Endothelium: Arteries and veins	Strong
		Glomerular capillaries	Variable
	Heart	Endothelium: Arteries, veins, capillaries	Strong
		Endocardium	Strong
		Myocardium	Perinuclear
10	Liver	Small Bile Ducts (lining cells)	Strong
		Endothelium: Arteries, veins	Strong
		Intertubular sinusoids	Negative

The specificity of the binding of anti-GAL antibodies was tested on sections of pig renal cortex by inhibition with 0.1 M melibiose, lactose, sucrose and glucose. Reactivity of the anti-GAL antibodies with proximal tubule brush borders was reduced to near background by preincubation of antibody with melibiose, but was not inhibited by the other saccharides.

20

## EXAMPLE 3

Hemagglutination of Pig RBC by Human Serum:Sugar Inhibition Studies

The methods used to investigate the hemagglutination of pig red blood cells (RBC's) by human serum was adapted from the methods described by Galili, J.Exp. Med. 160: 1579-81 (1984) and Severson, Immunol. 96: 785-789 (1966).

25

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## I. METHODS

A. Media/Solution Preparation

1. Human Serum Albumin (HSA) (CSL, Melbourne, Australia) (5mg/ml) was dissolved in PBS, filter sterilized, and stored at 4°C.

## 2. Preparation of sugars:

- 1M stock solutions of sugar were prepared by dissolving the amount indicated in 100ml of PBS. Sodium azide was added (0.02%) and solutions stored at 4°C.

$\alpha$ -Lactose (4-O- $\beta$ -D-galactopyranosyl- $\alpha$ -D-glucose	36.0g
D-(+)-galactose	18.0g
Stachyose ( $\alpha$ -D-gal-[1->6]- $\alpha$ -D-Glc-[1->2]- $\beta$ -D-Fru)	66.6g
Melibiose (6-O- $\alpha$ -D-galactopyranosyl- D-glucose)	34.2 g
Sucrose ( $\alpha$ -D-Glucopyranosyl $\beta$ -D-fructofuranoside)	34.2 g
D-(+)-Glucose	18.0 g
$\alpha$ -D-(+)-Fucose (6-Deoxy-D-galactopyranose)	16.4 g

All sugars were purchased from Sigma (St. Louis, Missouri, USA). Sugar solutions were diluted in PBS to the appropriate concentration as required.

B. Preparation of pig RBC' S

1. Heparinised pig blood (Animal Resources, Clayton, Australia) is centrifuged at 800 RPM for 10min to pellet the RBC.

2. The RBC pellet is washed by resuspension in PBS (10ml) and recentrifugation (repeated 3 times). After the final wash, the RBC pellet is resuspended in 10ml PBS.

3. A 0.5% solution of RBC' s is prepared by adding 50ul RBC solution (from step 2, above) to

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10 ml PBS containing 0.5g/100 ml  
of HSA.

C. Preparation of 96-well microtitre plates  
(Titretek, USA)

- 5 1. Add 25ul of PBS to each  
well.
- 10 2. Add 25ul of pooled human AB  
serum (CSL, Melbourne,  
Australia) to column 1 and  
serially dilute by removing 25ul  
from column 1 and adding to  
column 2, then repeating by  
sequentially removing and adding  
25ul from and to each well
- 15 across the plate, finally  
discarding 25ul from column 11  
and adding no serum to column  
12.
- 20 3. Add 25ul of sugar solution  
to each row in decreasing  
concentrations down rows. No  
sugar solution is added to the  
final row.
- 25 4. Incubate at 4°C overnight  
and then at 37°C for 30 min.
- 30 5. Add 50ul of 0.5% pig RBC to  
each well; vortex and incubate  
at room temperature for 2 hours.  
Determine agglutination  
visually.

II. RESULTS

Human serum caused the agglutination of pig RBC's  
at a titre of between 1/32-1/64, which is consistent with  
the presence of high levels of naturally occurring

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xenoantibody (NXAb) in human serum. To examine the specificity of the NXAb response, sugar inhibition studies were performed. Sugars such as melibiose, stachyose, galactose and fucose which have terminal galactose residues in the  $\alpha 1-6$  configuration were found to inhibit agglutination in the  $\mu\text{M}$  to  $\text{mM}$  range. Sugars with other structures, such as lactose and sucrose, were only inhibitory when very high concentrations were used. At these high concentrations, the observed effects are most probably non-specific, due, for example, to changes in osmolarity. Results are summarized below:

Pig RBC Hemagglutination by Human Serum: Sugar Inhibition

	<u>Sugar</u>	<u>Linkage</u>	<u>Inhibitory Concentration</u>
15	Melibiose	Gal $\alpha 1-6\text{Glc}$	$5 \times 10^{-6}\text{M}$
	Stachyose	Gal $\alpha 1-6\text{Gal}$	$2 \times 10^{-3}\text{M}$
	Galactose		$2 \times 10^{-3}\text{M}$
	Fucose	6-Deoxy- $\alpha$ -L-Gal	$1 \times 10^{-3}\text{M}$
	Lactose	Gal $\beta 1-4$ -Glc	$> 10^{-1}\text{M}$
	Sucrose	$\alpha$ -D-Glc- $\beta$ -D-Fruc	$> 10^{-1}\text{M}$

20

#### EXAMPLE 4

##### Inhibition of Human Serum-Induced Lysis of Porcine Cells by Sugars

The ability of human serum to cause the lysis of porcine cells was examined using both pig epithelial (PK<sub>1</sub>) and aortic endothelial (PAE's) cells, the isolation and culture of which is described in Example 2. Cell lysis was determined using either the  $^{51}\text{Chromium}$  release assay as described by Cerottini and Brunner, Nature New Biol. 237:272, 1972 or the Cytotox LDH release assay according to the manufacturer's instructions (Promega, USA).

#### I. METHODS

##### A. $^{51}\text{CR}$ Release Assay

##### 1. Preparation of Cells:

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- a) Trypsinize a confluent flask of cells. On average, approximately  $3 \times 10^6$  PAE's and approximately  $3 \times 10^7$  PK<sub>1</sub> cells are obtained per 10 ml flask. About  $1 \times 10^5$  cells are required for each well in the <sup>51</sup>CR Release Assay.

- b) Wash cells 4 times in 10 ml RPMI (no FCS); spin 1200 rpm for 5 min.

- c) Resuspend cells in 100  $\mu$ l RPMI (with 10% heat-inactivated FCS; see below).

10            2.    Labelling Cells with <sup>51</sup> CR:

- a) Combine in a 10 ml tube: Cells in 195  $\mu$ l RPMI/10% FCS (heat inactivated); 5  $\mu$ l 51 CR (120  $\mu$ Ci).

- b) Incubate at 37°C for 2 hr.

- c) Add 2 ml RPMI/10% FCS (heat  
15 inactivated).

- d) Centrifuge cells through a layer of FCS (heat inactivated) to remove excess label.

- e) Gently overlay the labelled cells onto a 4 ml cushion of FCS using a Pasteur pipette.

- 20 f) Centrifuge at 700g for 5 min. at 4°C.

- g) Remove supernatant taking care not to disturb the cell pellet.

- h) Resuspend pellet in RPMI/10% FCS (heat inactivated) at about  $3 \times 10^7$  cells/ml.

25                    3.    Assay Conditions:

- a) For PAE's, rabbit complement was used as the complement source, since the <sup>51</sup> CR-release assay was not sufficiently sensitive to detect lysis when human complement, a less "active" source, was used. In

- 30 contrast, with the LDH assay, which is significantly more  
sensitive, normal human serum (NHS) was used as the  
source of complement.

- b) To each test well of a 96-well V bottom plate, add:

- 35 - 100  $\mu$ l labelled cells

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- of final)
- 10-50  $\mu$ l NHS (heat inactivated) (5-25%
  - Complement:
    - PAE's: 50  $\mu$ l absorbed
    - PK<sub>1</sub>: 10-40  $\mu$ l NHS (5-25% of
- 5 rabbit complement (25% final)
- final)
- 50  $\mu$ l antibody (total anti-GAL (IgG +
  - IgM, anti-GAL IgG, anti-GAL antibody-depleted serum, or
- 10 anti-GAL antibody-depleted IgG)
- c) Adjust volume to 200  $\mu$ l with
  - RPMI/10% FCS (heat inactivated) if required
  - d) Incubate plates at 37°C for 3 hr.
  - e) Centrifuge plates at 1000 rpm for 5
- 15 min to pellet cells
- f) Remove 100  $\mu$ l of supernatant from
  - each well and transfer to a gamma counter tube
  - g) Add 3 ml scintillation fluid and
  - measure <sup>51</sup>CR release using a gamma counter (Packard
- 20 Instrument Company, Illinois, USA)
- (To determine maximum release, add 100
- $\mu$ l 8% Triton X-100 made up in RPMI/10% FCS (heat
- inactivated) to 100  $\mu$ l labelled cells)
- (Note: Each reaction is set up in
- 25 quadruplicate)
4. Calculation of % Lysis:
- $$\% \text{ Lysis} = \frac{\text{Experimental cpm} - \text{Spontaneous Release cpm}}{\text{Max. Release cpm} - \text{Spontaneous Release cpm}} \times 100$$
5. Sugar Inhibition of Complement-Induced Cell
- 30 Cytotoxicity:
- In a 96-well test plate, mix the following:
- 50  $\mu$ l labelled cells
  - 50  $\mu$ l complement
- (PAE's: pig spleen cell absorbed complement; PK<sub>1</sub>'s: NHS)
- x  $\mu$ l sugar (final concentration of sugar:
- 35 10<sup>-1</sup> to 10<sup>-3</sup> M)
- y  $\mu$ l NHS (heat inactivated) - final
  - concentration 5-20%)
  - make volume to 200  $\mu$ l with RPMI



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Plate Layout:

		<u>Plate 1</u>		<u>Plate 2</u>	
		5%	10%	15%	20%
Rows:		1-4		5-8	1-4 5-8
5	Columns:	1. Spontaneous Release 2. Maximum Release 3. Melibiose 4. Lactose			

B. LDH Release Assay

## 10 1. General Procedures:

a) Prepare cells as for <sup>51</sup>CR Release assay, and labeled with LDH as per the manufacturer's instructions (Cytotox non-radioactive LDH release assay, Promega, USA)

## 15 b) To each well of a 96-well plate add (each reaction set up in quadruplicate):

- 25 µl labeled cells
- 5-20 µl NHS
- x µl sugar (final concentration of sugar: 10<sup>-1</sup> to 10<sup>-3</sup> M)
- RPMI/10% FCS (heat inactivated), to total volume of 100 µl

c) Incubate plates at 37°C for 3 hr.

d) Centrifuge plates at 1500 rpm for 5 min.

25 e) Remove 50 µl supernatant from each well (taking care not to remove any cells) and transfer to ELISA plate containing 50 µl substrate mix (prepared according to manufacturer's instructions

30 f) Cover tray and incubate in the dark at room temperature for 30 min.

g) Add 50 µl stop solution to each well using multichannel pipette

h) Read absorbance at 492 nm.

## 2. Controls:

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a) Spontaneous release (no antibody or complement)

- 25  $\mu$ l labeled cells
- 75  $\mu$ l RPMI/10% FCS (heat inactivated)

5 b) Maximum release

- 25  $\mu$ l labeled cells
- 50  $\mu$ l 16% Triton X-100
- 25  $\mu$ l RPMI/10% FCS (heat inactivated)

3. Calculation of % Lysis: % Lysis =

10 
$$\frac{\text{Experimental release} - (\text{Spontaneous release cpm} + \text{sugar cpm})}{\text{Maximum release} - (\text{Spontaneous release cpm} + \text{sugar cpm})} \times 100$$

Maximum release - (Spontaneous release cpm + sugar cpm)

4. Experimental Design:

Plate 1

15 Columns: 1. spontaneous release  
2. maximum release  
3. 5% serum  
4. 10% serum  
5. 25% serum  
20 6. RF10 alone

Rows: 1-4: cells + no sugar  
5-8: no cells + no sugar

Plate 2 melibiose  
Plate 3 galactose  
Plate 4 lactose  
Plate 5 sucrose

25 Plates 6-9 same as plates 2-5 but no cells added

Sugar Conc.  
Columns: 1-2  $1 \times 10^{-1}M$   
3-4  $5 \times 10^{-2}M$   
5-6  $1 \times 10^{-2}M$   
7-8  $5 \times 10^{-3}M$   
9-10  $2 \times 10^{-3}M$   
30 11-12  $1 \times 10^{-3}M$

Rows: 1-2 0% serum  
3-4 5% serum  
5-6 10% serum  
7-8 25% serum

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5. Preparation of Pig Spleen-Absorbed Rabbit Complement:

- a) Cut pig spleen (obtained from local abattoir) into small pieces and prepare a single-cell suspension by passage through a fine metal sieve
- 5 b) Pellet cells by centrifugation at 700g, 7 min. at 4°C
- c) Resuspend cell pellet in RPMI/10% FCS and repeat centrifugation
- d) Resuspend in RPMI/10% FCS/10%  
10 dimethylsulfoxide (DMSO)
- e) Count cells and store frozen aliquots ( $3 \times 10^9$  cells/aliquot)  
- use one aliquot for each absorption
- f) For absorption, thaw and centrifuge at 600g, 5  
15 min. at 4°C and remove the supernatant containing the DMSO
- g) Wash two times with RPMI/10% FCS (10 ml)
- h) Resuspend the cell pellet in rabbit complement; mix (rotary wheel) 2 hr. at 4°C
- 20 i) Centrifuge 600g, 5 min. at 4°C and remove the supernatant containing the rabbit complement; store at 4°C

II. RESULTS

Comparable results were obtained with both cell  
25 types (PAE's and PK<sub>1</sub>'s) using both lysis assays. The results of a typical lysis experiment are represented in Figure 2, in which the lysis of PAE's by human serum and by purified anti-GAL antibodies was determined using the <sup>51</sup>CR release assay. Comparable results were also obtained  
30 with PK<sub>1</sub> cells using the <sup>51</sup>CR release assay and with both cell lines using the LDH release assay. The results of these assays can be summarized as follows:

1. Xenoantibodies (NXAb) in human serum in the presence of complement are capable of lysing porcine

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cells. Lysis increases with increasing concentrations of serum.

2. Pre-absorption of NHS with pig spleen cells (which removes the NXAb): No lysis.
- 5 3. Use of heat-inactivated complement: No lysis.
4. Use of NHS depleted of anti-GAL antibodies: No lysis.
5. Use of purified total anti-GAL antibodies  
10 (IgG + IgM): Lysis.
6. Use of purified anti-GAL IgG: No lysis.
7. Use of purified total anti-GAL antibodies (IgG + IgM) and dithiothreitol (DTT): No lysis. (DTT is a reducing agent that disrupts the multimeric structure  
15 of IgM antibodies without affecting IgG.)

Together these results demonstrate that the anti-GAL antibodies are responsible for the observed lysis. Purified anti-GAL IgG and DTT-treated total (IgG + IgM) anti-GAL antibodies failed to elicit lysis, indicating  
20 that IgM, but not IgG, antibodies are causative agents in this system. Preliminary attempts to verify this observation using purified IgM prepared either in crude form by euglobulin fractionation or by  $\alpha$ -IgM affinity chromatography were unsuccessful. The inventors believe  
25 this reflects inactivation of the IgM during preparation, rather than a true reflection of the capacity of anti-GAL IgM to cause lysis of porcine cells. heat inactivation of the complement prevented lysis, indicating that lysis of porcine cells is a complement-dependent phenomenon.

30 The effect of adding the disaccharide sugars melibiose (Gal  $\alpha$ 1 $\rightarrow$  6 Gal) and lactose (Gal  $\beta$ 1 $\rightarrow$  4 Glu) on the lysis of PAE's by human serum was assessed using the Cytotox non-radioactive LDH release assay. PAE's were incubated in the presence of 50% human serum as the

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source of xenoantibody and complement, together with various concentrations of each sugar (1mM to 100mM). Under these conditions, melibiose, which has the Gal  $\alpha$ 1 $\rightarrow$  6 Gal configuration, but not lactose, which has the terminal Gal moiety by in a  $\beta$ 1 $\rightarrow$  4 configuration, protected the pig cells from lysis.

#### EXAMPLE 5

##### Inhibition of Human Serum-Induced Damage to Rat Hearts by Sugars

The Langendorf isolated perfused ex vivo heart model was used to further demonstrate the involvement of anti-GAL xenoantibodies in hyperacute rejection.

#### I. METHODS

##### A. Preparation and storage of Human Plasma

1. Centrifuge fresh human blood at 3000 rpm, 10 min., 4°C to remove red blood cells (RBC's)
2. Remove the plasma
3. Centrifuge the plasma at 10,000 rpm, 10 min. 4°C to remove any remaining cells; decant the plasma
4. Add 2.5 ml of 0.1M EDTA pH 7.30 for every 50 ml of plasma
5. Store 50 ml aliquots at -70°C
6. For heat-inactivated plasma, heat at 56°C for 60 min., then centrifuge at 2,500 rpm for 10 min.

##### B. Assessment of Complement Activity

Before being used in the ex vivo model, both heat inactivated and control plasma was tested for complement activity. Classical complement activity was determined by hemolysis using sensitized sheep RBC's as described by Harrison and Lachman, In: Weir et al. (eds.), Handbook of Experimental Immunology and Immunochemistry, 4th Ed., Blackwell scientific Publications (1986). Alternative complement pathway activity was determined using the

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rabbit hemolytic assay as described by Serrais et al., J. Immunol. Meth. 140: 93-100 (1991). The assay was performed in buffer containing EGTA and  $MgCl_2$ . The EGTA chelates the  $Ca^{++}$ , thus inhibiting the classical pathway.

- 5 The  $Mg^{++}$  is required for activation and assembly of CdbBb, the alternative pathway C3 convertase.

C. Preparation of Plasma for Heart Perfusions

- Plasma prepared from different blood packs is thawed at 37°C, pooled and filtered (100  $\mu m$  steel mesh,  
10 8.0  $\mu m$  and 4.5  $\mu m$  Millipore filters, sequentially).  $CaCl_2$  is added at 0.58 mg/ml plasma, and the plasma kept on ice until ready for perfusion.

D. Ex Vivo Isolated Perfused Rodent Heart Model

1. Anesthetize rats with Nembutal (1  $\mu l$  sodium  
15 pentobarbitone (60 mg/ml)/g body weight) and mice with ether.
2. Surgically expose the heart and inject heparin (Porcine Mucous, 10,000 U/ml) into the femoral vein (rats: 0.3 ml injected).
- 20 3. Remove heart and place in ice-cold Krebs-Henseleit buffer containing heparin (0.2 ml/50 ml buffer.

Krebs-Henseleit buffer:

- 119 mM NaCl
- 25 mM  $NaHCO_3$
- 25 - 4.6 mM KCl
- 1.2 mM  $MgSO_4 \cdot 7H_2O$
- 1.3 mM  $CaCl_2 \cdot 2H_2O$
- 1.2 mM  $KH_2PO_4$
- 11 mM glucose
- 30 - 0.25% (v/v) BSA
- Adjust to pH 7.4; store at 4°C

4. Connect aorta to the canula of the Langendorff perfusion apparatus and tie firmly. The apparatus was assembled by the present inventors according to  
35 experimental requirements of the Langendorff heart model as described in Doring & Dehner, The Isolated Perfused Heart According to Langendorff, Bionestechnik-Verlag March GmbH, D7806, West Germany.

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5. Perfuse with Krebs-Henseleit buffer (made fresh each day), which is gassed continuously with carbogen (95% O<sub>2</sub>, 5% CO<sub>2</sub>) at a pressure of 100 mmHg, at 37°C.
- 5        6. Attach a hook, connected to a transducer (Physiograph MK-111-S, Narco Bio-Systems) to the apex of the heart.
7. Perfuse heart for 20 min. with Krebs-Henseleit buffer to enable heart to stabilize (reservoir  
10 volume: 270 ml).
8. Add plasma (pre-warmed to 37°C) as follows:
  - at 20 min. - add 10 ml plasma (= 5% plasma)
  - at 25 min. - add 10 ml plasma (= 9 % plasma)
  - at 30 min. - add 10 ml plasma (= 13 % plasma)
- 15        9. Monitor heart for a further 30 min. and record heart flow and contraction rate.

E. Sugar perfusion

1. Stabilize heart in Krebs Henseleit buffer for 30 min. as described above.
- 20        2. Add 2.5 ml of 1.08 M stock sugar solution to reservoir; total volume = 270 ml; final sugar concentration = 10mM.
3. Allow heart to restabilize for 10 min, then add plasma (control or heat inactivated) as per the  
25 schedule described above.
4. Record heart beat and flow rate.

F. Large-Scale Preparation of anti-GAL antibody-Depleted Plasma

- (all manipulations are performed at 4°C)
- 30        1. Start with 200 ml freshly prepared human plasma; 100 ml is subject to depletion; 100 ml is used as an untreated control from the sam patient drawn on the same day; store at 4°C.

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2. Filter the plasma sequentially through a 100  $\mu\text{m}$ , 8  $\mu\text{m}$  metal sieves and finally through a 0.45  $\mu\text{m}$  Millipore filter; dilute to 1000 ml with PBS, pH 8.0.
3. Concentrate to 200 ml using an Amicon spiral  
5 wound cartridge (removes salt).
4. Equilibrate melibiose sepharose column (40 ml) with PBS, pH 8.0 (10 column volumes).
5. Passage the plasma through the melibiose sepharose column; collect the run-through and store at -  
10 70°C (=partially depleted plasma).
6. Wash column with PBS, pH 8.0 (10 column volumes) until the O.D. (280nm) of the eluate is approximately zero.
7. Combine the partially depleted plasma and the  
15 eluate from the wash; concentrate to 200 ml (Amicon spiral concentrator).
8. Elute the anti-GAL antibody fraction with 4M guanidinium HCl pH 6.4 (2 column volumes).
9. Regenerate the column with PBS (10 column  
20 volumes).
10. Repeat the entire process an additional two times, i.e., repassage plasma through the melibiose column, wash, elute the anti-GAL antibody fraction and regenerate column.
- 25 11. For the anti-GAL antibody-depleted fraction:
  - combine the eluate from the melibiose sepharose column with run-through from the final wash
  - adjust the volume to 5 liters with Krebs Henseleit buffer and add EDTA to 10 mM; adjust pH  
30 to 7.0
  - concentrate back to original volume (Amicon spiral concentrator); aliquot (35 ml) and store at -70°C
12. For the anti-Gal antibody fraction:



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- combine the eluted anti-GAL antibody fractions, dilute to 5 liters with Krebs Henseleit buffer and add EDTA to 10 mM

- concentrate back to 10 ml (Amicon 5 spiral concentrator); aliquot (1 ml) and store at -70°C

13. The anti-GAL antibody-depleted fraction and the purified anti-GAL antibody fraction are tested for

a) Anti-GAL reactivity: Use as primary reagents to stain porcine cells (PK<sub>1</sub>'s). Detect 10 staining as described in Example 2, above. Analyze stained samples using a FACScan II (Becton Dickinson), according to the manufacturer's instructions.

b) Protein content: Determine using the colorimetric method of Bradford, Anal. Biochem. 72: 15 248-54 (1976), with purified human IgG as the standard.

c) Electrolyte concentration: On the day of the perfusion, the anti-GAL antibody depleted plasma is also tested to determine the calcium, magnesium and potassium levels using an electrolyte autoanalyser 20 (Olympus); the levels of each are adjusted to normal as required.

## II. RESULTS

Rat hearts were connected to the Langendorf apparatus and then stabilized by perfusion with Krebs 25 Henseleit buffer for 10 min., and then a further 10 min. with the same buffer containing either melibiose or lactose (10mM). Human plasma was then added in stages as described above to a final concentration of 13 % and the effect of the added sugar on cardiac function was 30 assessed. The parameters measured were heart rate, amplitude (strength) of contraction and output (Figure 3).

In the presence of human serum alone (lower trace), the heart essentially stopped beating within

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minutes. The same result was obtained if lactose was added. In the presence of melibiose (upper trace) or anti-GAL antibody-depleted plasma, however, the heart was able to maintain a strong beat. When the purified anti-  
5 GAL antibody was added back to the anti-GAL antibody-depleted plasma, the heart again stopped beating within minutes.

## EXAMPLE 6

Characterization of the Porcine  $\alpha$ -1,3-GalT Gene

10 cDNA's encoding porcine  $\alpha$ -1,3-GalT were generated by Polymerase Chain Reaction (PCR) technology. Total RNA of pig liver was isolated by homogenizing liver slices in 7M guanidinium thiocyanate, as described by Chomczynski & Sacchi, Anal. Biochem 162, 156-159 (1987); Sambrook et  
15 al., Molecular Cloning: A Laboratory Manual (2nd Edition), Cold Spring Harbor Laboratory Press (1989). Sixteen  $\mu$ g of the RNA, together with 1 $\mu$ g oligo dT primer, were heat denatured for 5 minutes at 65°C prior to being transcribed into cDNA using avian myeloblastosis virus  
20 (AMV) reverse transcriptase in a 100 $\mu$ l reaction carried out at 37°C for 90 minutes. Three  $\mu$ l of the cDNA synthesis reaction was used in the subsequent PCR amplifications. General procedures used for generation of cDNA are provided in Sambrook et al (1989), supra.  
25 Primers for PCR were synthesized using phosphoramidite technology, on an Applied Biosystems DNA synthesizer. The sequence of the PCR primers was based on identifying conserved regions within the published sequences for murine and bovine  $\alpha$ -1,3-GalT genes.  
30 Joiazze et al., J. Biol. Chem 264: 14290-97 (1989); Joiazze et al., Biol. Chem 267: 5534-5541 (1992). All primers were synthesized with EcoR1 linkers at the 5' end for ease of cloning. In the following listing of the primers used in the present study, nucleotide positions

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varying between bovine and murine sequences are single-underlined; nucleotide positions varying between bovine and human sequences are double-underlined:

Exon 2 primer (forward):

5 5'-GTGAATTCAGCCCTGCCTCCTTCTGCAG-3'

(SEQ ID NO: 1)

Designation: GTE2F -- 28-mer

- 1 difference b/w bovine & murine

- no sequence available for human exon 2

10 Exon 4 primer (forward):

5'-GTGAATTCAGGAGAAAATAATGAATGTC-3'

(SEQ ID NO: 2)

Designation: GTE4F -- 28-mer

- no differences b/w bovine, murine & human

15 Exon 9 primer (reverse):

5'-GTGAATTCGGGATCTGCCTTGTACCACC-3'

(SEQ ID NO: 3)

Designation: GTE9R -- 28-mer

- 3 differences b/w bovine & murine

20 - 1 difference b/w bovine & human

3'-UTR primer (reverse):

5'-GTGAATTCGAAATCACTGCGAATTTACA-3'

(SEQ ID NO: 4)

Designation: GT3UR -- 28-mer

25 - no differences b/w bovine & murine

- no differences b/w bovine & human

Exon 9 primer (forward):

5'-AGGAATTCAGCATGATGCGCATGAAGAC-3'

(SEQ ID NO: 5)

30 Designation: GTE9F -- 28-mer

- no differences b/w bovine & murine

- 3 differences b/w bovine & human

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PolyA primer (reverse): 5'-TTGAATTCTTTTTTTTTTTTV\*N\*\*-3'

(SEQ ID NO: 6) \* V = A or C or G; \*\* N = A or C or G  
or T

(primer includes all nucleotide variants  
5 for V and N)

Designation: APATR -- 23-mer

The PCR conditions used to generate porcine  $\alpha$ -1,3-GalT  
cDNA fragments were as

follows:

- 10 1) For GTE2F + GTE9R and GTE4F + GTE9R: heat to 94°C (60  
seconds); then proceed with 35 reiterations (cycles) of  
the following three steps: (1) 94°C, 40 seconds, (2)  
57°C, 50 seconds, and (3) 72°C, 80 seconds.  
2) For GTE9F + GT3UR: heat to 94°C (120 seconds); then  
15 proceed with 35 cycles of: (1) 94°C, 40 seconds, (2)  
48°C, 45 seconds, and (3) 72°C, 60 seconds.

The PCR fragments were subcloned into EcoR1-  
restricted pBluescript II KS+ (Stratagene, Cat, # 2  
12206) and the DNA sequence was determined using the  
20 chain termination method. The DNA sequence was assembled  
and analyzed using DNASIS-Mac v2.01 (Hitachi)

The nucleotide sequence of porcine  $\alpha$ -1,3-GalT (SEQ  
ID NO: 7) and the derived amino acid sequence (SEQ ID NO:  
10) of the enzyme are shown in Figures 4 and 5. A single  
25 large open reading frame extends from the initiating  
methionine at nucleotide 91 to a stop codon located at  
nucleotide 1204. The sequence surrounding the putative  
initiating methionine conforms to the consensus  
eukaryotic initiation sequence. Kozak, Cell 44, 283-92  
30 (1986).

The porcine cDNA sequence is compared to the  
corresponding murine (SEQ ID NO: 9) and bovine (SEQ ID  
NO: 8) sequences in Figure 4. The locations of introns  
within the murine gene are also shown. Joizazze et al.,  
35 J. Biol. Chem 267: 5534 (1992). This alignment

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demonstrates that exon 3, located within the 5' untranslated region of the mouse gene, is not found in either the porcine or bovine cDNAs. The overall sequence identities between the coding sequences are as follows:

5           a) pig compared to mouse:- 75.02% (exon 3 not considered)

          b) pig compared to bovine:- 85.15%

          The amino acid sequences of the porcine (SEQ ID NO: 10), murine (SEQ ID NO: 12) and bovine (SEQ ID NO: 10 11)  $\alpha$ -1,3-GalT enzymes are depicted in Figure 5. The locations of introns are also shown, based on their positions within the mouse gene (Joziassse et al., 1992). This alignment illustrates that the overall amino acid homologies are:

15           a) pig compared to mouse: 71.98%

          b) pig compared to bovine: 82.87%

          c) bovine compared to mouse: 73.72%

#### EXAMPLE 7

##### Identification of Potential Sites to Interrupt the $\alpha$ -1-3-GalT Gene

20           The present inventors' choice of a site for interrupting the  $\alpha$ -1,3-GalT gene has been influenced by several characteristics of the gene and its expression. In particular, several mRNAs for  $\alpha$ -1,3-GalT have been detected in the mouse. Joziassse et al., J. Biol. Chem. 25 267: 5534 (1992). These mRNAs are products of alternative splicing events in which exons 5 and/or 6 may be deleted. Hence, these exons are not appropriate interruption sites in the mouse, since a transcript encoding a functional  $\alpha$ -1,3-GalT enzyme presumably could 30 be formed when exons 5 or 6 are spliced out. Moreover, the present inventors have isolated two different classes of  $\alpha$ -1,3-GalT cDNA clones from the pig - one that includes exon 5 and one with exon 5 deleted. It is possible that mRNA's with and without exon 6 are also

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formed by alternative splicing in the pig. Thus, for initial experiments the present inventors have not chosen these exons as sites for interruption.

Insertion of an interrupting-DNA fragment into  
5 exon 4 (which encodes the cytoplasmic NH<sub>2</sub>-terminal domain and the membrane-anchoring domain; see Figure 5) would disturb production of a transcript encoding an active  $\alpha$ -1,3-GalT. Hence this exon is an appropriate site to  
10 disrupt the  $\alpha$ -1,3-GalT gene. Similarly, exons 7 and 8, which encode the NH<sub>2</sub>-terminal region of the catalytic domain, are suitable disruption sites. Insertion of a  
interrupting DNA fragment within these exons would prevent the synthesis of an active catalytic domain.

A preferred site for interrupting the mouse gene  
15 is located at a Sall site found within exon 9 of the mouse  $\alpha$ -1,3-GalT gene, at codons 221 + 222 (see Figure 5). This site is positioned 150 amino acids from the COOH-terminus, within the catalytic domain. The mouse  
gene within the present inventors' constructs for  
20 homologous recombination is interrupted at this Sall site. The amino acids encoded by nucleotides at this Sall site are conserved in the pig and bovine sequences, although the Sall site itself is not. Construction of a  
Sall site at this position in the pig gene (e.g., by *in*  
25 *vitro* mutagenesis) provides a useful construct to inactivate the gene.

#### EXAMPLE 8

##### Choice of a DNA Fragment to Interrupt the $\alpha$ -1,3-GalT Gene

The present inventors have used both the neomycin

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resistance ( $\text{neo}^R$ ) gene and the hygromycin resistance gene ( $\text{hyg}^R$ ) to interrupt the  $\alpha$ -1,3-GalT gene. In one set of "knockout" constructs the  $\text{neo}^R$  and  $\text{hyg}^R$  genes are linked to the murine phosphoglycerate kinase (PGK) promoter  
5 (Adra et al., Gene 60: 65-74 (1987) and are both bordered by polylinker sequences that include restriction sites for EcoRV and ClaI.

In another construct, expression of the  $\text{neo}^R$  gene is directed by an altered polyoma virus promoter (PMCl;  
10 Thomas and Cappechi, cell 51: 503-12 (1987)). In this construct the present inventors have addressed the problem of including an antibiotic resistance gene within the genome of transplant organs. That is, in some circumstances it may not be desirable to have genes  
15 conferring resistance to antibiotics present in the organ to be transplanted. The FLP/FRT recombinase system of yeast has been used to eliminate the  $\text{neo}^R$  gene from the sequence that interrupts the  $\alpha$ -1,3-GalT gene.

In a construct of the present invention, the  $\text{neo}^R$   
20 gene is bordered at both the 5' and 3' ends by FRT DNA elements. In addition, stop codons for each of three reading frames have been inserted 3' to the  $\text{neo}^R$  gene, and these stop codons, together with a single FRT sequence, will remain within the  $\alpha$ -1,3-GalT gene after  
25 the  $\text{neo}^R$  gene has been excised by FLP. Targeted cells carrying a genomic copy of the neo gene flanked by direct repeats of the FRT could be supplied with FLP recombinase in two ways:

1) Introduction into cells of partially purified  
30 FLP protein:

FLP protein (0.1 - 10  $\mu\text{g}$ ) is introduced ("transfected") into approximately  $10^7$  cells using standard electroporation conditions. The cells are plated out into gelatinized tissue culture dishes in

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appropriate medium, at a sufficient dilution to result in individual colonies. Approximately 200 of these colonies are then picked for further analysis.

2) Transfection with plasmids containing the FLP  
5 gene:

A plasmid containing the FLP gene under control of a promoter able to drive FLP expression, e.g., the human interferon-inducible 6-16 promoter, is constructed according to standard methods. Porter et al., EMBO J. 7:  
10 85 (1988). Approximately 10  $\mu$ g of FLP expression plasmid is transfected into approximately  $10^7$  cells using standard electroporation conditions. With a plasmid containing the human 6-16 promoter, interferon is added at approximately 500 units/ml, in order to induce  
15 expression of FLP. The cells are then treated as in (1), above.

The procedure to knock out the  $\alpha$ -1,3-GalT gene in ES cells using an FRT-containing construct is:

- a) electroporate the complete construct into ES  
20 cells
- b) select neo<sup>R</sup> cells, and identify those ES cells having an interrupted  $\alpha$ -1,3-GalT gene
- c) delete the neo<sup>R</sup> gene using FLP recombinase, as described above; cells are tested for the excision  
25 event as follows:

First, samples of each selected cell line are tested for the absence of the neo<sup>R</sup> gene by treatment with the chemical G418. The cells will die in the presence of approximately 200  $\mu$ g/ml G418 unless the neo<sup>R</sup> gene is still  
30 present in the genome. Cell lines that are G418 sensitive are then tested further to confirm that excision of neo<sup>R</sup> has occurred. This is done by Southern analysis or PCR analysis, both described in Sambrook et al. (1989). For Southern analysis, genomic DNA is  
35 isolated from the cells, digested with an appropriate



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restriction enzyme, subjected to agarose gel electrophoresis, and the digested DNA transferred to a membrane. The DNA is hybridized with a labeled probe, the label is detected (e.g., with X-ray film or color development), and the pattern of bands indicates whether or not an excision event had occurred in the cell line. For PCR analysis, genomic DNA is isolated from the cells and subjected to PCR reaction with suitable oligonucleotide primers.

- 10           d) following confirmation of neo<sup>R</sup> excision, the manipulated ES cells or PGC's are used to generate chimeric animals.

#### EXAMPLE 9

##### Preparation of DNA Constructs to Interrupt the $\alpha$ -1,3-GalT

##### 15 Gene in Mice

- Gene targeting (homologous recombination) is more efficient if the cloned cDNA fragments used for targeting are isolated from the cell line which is used for the gene knockout (i.e., the DNA is "isogeneic").
- 20 Accordingly, DNA was isolated from the E14 ES cell line (Hooper et al., Nature 326: 292-95 (1987)) and used to construct a mouse genomic library. The DNA was digested partially with the restriction enzyme Sau 3A, and fragments 12 kb - 20 kb in size were isolated by glycerol
- 25 gradient fractionation. The size-fractionated DNA was ligated into the Bam H1 site of  $\lambda$ EMBL3 (Sambrook et al. 1989, supra), and packaged *in vitro* to form lambda phage particles. The lambda library was plated by infection of E. coli strain PMC103 host cells (Doherty et al., Gene
- 30 124: 29-35 (1993)) at a density of  $4 \times 10^4$  phage per plate. A bovine cDNA clone, about 900 bp in length and containing a portion of the  $\alpha$ -1,3-GalT gene corresponding to exons 7 - 9, was used to probe a total of  $5.6 \times 10^5$  independent recombinant phage. Four overlapping clones

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containing  $\alpha$ -1,3-GalT gene sequences were isolated and purified. The Sall restriction sites within these clones were mapped (Figure 6), and the 4.0kb, 5.5kb, 11kb and 12kb Sall fragments from two of the clones ( $\lambda$ 3 and  $\lambda$ 5) were subcloned into pBlueScript KS+ (Stratagene) or pUBS (pUC19 carrying the pBlueScript KS+ polylinker) to facilitate further detailed mapping of restriction sites.

These four subclones (designated p $\alpha$ GT-S4.0, p $\alpha$ GT-S5.5, p $\alpha$ GT-S11 and p $\alpha$ GT-S13) were mapped for restriction sites with restriction enzymes BamHI, EcoRI, HindIII, XbaI, XhoI, KpnI, SacI, SacII, EcoRV, PstI, SmaI, NotI and BglII. p $\alpha$ GT-S4.0 and p $\alpha$ GT-S5.5 were also checked for PvuI, PvuII, NdeI and SphI restriction sites. Detailed restriction maps of the 4 subclones were drawn from these data (Figures 7-12).

On the basis of these maps a knockout strategy was conceived. Basically the strategy is to insert a resistance gene (either neo<sup>R</sup> or hyg<sup>R</sup>) into the Sall site which lies within Exon 9. The knockout construct carries the 4.0 and 5.5kb Sall fragments from p $\alpha$ GT-S4.0 and p $\alpha$ GT-S5.5 which flank the Exon 9 Sall site (Figure 13). Screening for homologous recombination events then can be carried out using a DNA fragment representing the genomic region but lying outside the DNA included in the knockout construct, i.e., outside the 9.5kb covered by p $\alpha$ GT-S4.0 and p $\alpha$ GT-S5.5. A 0.7kb EcoRI/XmnI fragment from p $\alpha$ GT-S11 is used to screen Southern blots of BglII digested ES cell DNA for homologous recombinant events. An 8.3kb band should appear on these Southern blots when the uninterrupted  $\alpha$ 1,3-GalT gene is probed with this EcoRI/XmnI fragment (Figure 14). Insertion of the neo<sup>R</sup> gene after a homologous recombination event will give rise to a 6.4kb band, due to the presence of a BglII site just flanking the Exon 9 Sall site within the knockout

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construct. Thus the presence of the 6.4kb band is diagnostic for a homologous recombination event.

To carry out this strategy, the present inventors prepared a series of knockout constructs. The generation  
5 of one such construct is outlined in detail in Figure 15. The vector p $\alpha$ GT-S5.5, which carries the 5.5kb fragment immediately 3' to the Exon 9 SalI site, was chosen as the starting vector. p $\alpha$ GT-S5.5 was digested with EcoRV and ClaI, generating a vector with a blunt end and a ClaI  
10 compatible end. A 1.3kb fragment carrying the PMC1 promoter-driven neo<sup>R</sup> gene flanked by FRT sites was excised from plasmid pNeo2FRT (previously constructed by the present inventors) by digesting with BamHI, filling in the restriction site and then digesting with ClaI to  
15 generate a fragment with one blunt end and one ClaI compatible end. The nucleotide sequence of this 1.3kb fragment is provided in Figure 16 (SEQ ID NO: 13). This fragment was then ligated into the ClaI/EcoRV digested p $\alpha$ GT-S5.5, the ligation mix transformed and colonies  
20 screened for recombinants. One colony was recovered that contained the Neo<sup>R</sup> fragment inserted into the EcoRV/ClaI of p $\alpha$ GT-S5.5, based on the restriction pattern after digestion with diagnostic restriction enzymes ClaI, EcoRV, XbaI and EcoRI. This construct was designated  
25 PNeo $\alpha$ GT6.8.

pNeo $\alpha$ GT6.8 was digested with SmaI, generating a vector with blunt ends. The 4.0kb SalI fragment was excised from p $\alpha$ GT-S4.0 and the ends filled. This  
30 fragment was then ligated into the SmaI digested p $\alpha$ GT-S5.5, the ligation mix transformed and colonies screened for recombinants. Four colonies were recovered which contained the 4.0kb SalI fragment inserted into the SmaI sites of pNeo $\alpha$ GT6.8 with the 5' portion of Exon 9 lying near the 3' portion of the exon in the nearby SalI 5.5kb  
35 fragment. The identity and orientation of the insert was

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confirmed by the restriction pattern after digestion with diagnostic restriction enzymes XbaI, EcoRI, HindIII, BamHI, EcoRV and others. This construct was designated pNeoαGT10.8.

- 5 pNeoαGT10.8 was digested with ClaI, generating a vector with ClaI compatible ends. Two complementary oligomers were synthesized that, when annealed, generated a linker containing translation termination codons in all three reading frames and a BglII site. The linker has
- 10 ClaI compatible ends. The linker was ligated into the ClaI digested pNeoαGT10.8, the ligation mix transformed and colonies screened for recombinants. Many colonies were recovered that contained the linker inserted into the ClaI sites within pNeoαGT10.8 based on the
- 15 restriction pattern after digestion with diagnostic restriction enzymes BglII, Cla and BglII/NotI. This construct has been sequenced to confirm the identity, copy number and orientation of the insert. This construct is called pNeoαGT10.8B (Figure 17).

20

## EXAMPLE 10

ES Cells - General Materials and MethodsWorking Conditions

- Procedures for the isolation and culturing of all cell lines (embryonic stem, primordial germ and fetal
- 25 fibroblast cell lines) require aseptic conditions to prevent growth of contaminating organisms:

1. All laboratory bench tops and equipment are wiped down with 70% ethanol prior to use.
2. All surgical instruments are autoclaved prior to
- 30 use.
3. Water for media preparation and cleaning of glassware is of high quality (e.g., Milli-Q water, prepared by passage through a Milli-Q ultrapure water system (Millipore)).

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4. Glassware is either dry-heat sterilized or autoclaved following extensive cleaning in Milli-Q water before use.

5. All tissue culture work is carried out under laminar flow conditions (Hepa filtered horizontal laminar flow workstation).

6. All media are filter sterilized (22 $\mu$ m disposable filter) prior to use.

7. Antibiotics are used to minimize the risk of bacterial contamination (Penicillin, Streptomycin and Gentamicin for bacteria; Nystatin for fungi).

#### Media/Solution Preparation

##### DULBECCOS MODIFIED EAGLE MEDIUM (DMEM)

- 10.0g DMEM powder- Gibco  
15 (the low-glucose or high-glucose formulation, with or without pyruvate, may be used; L-glutamine is included)  
1.0 liter Milli-Q-Water  
3.7g NaHCO<sub>3</sub>  
Stir slowly until dissolved  
20 Adjust pH ~ 7.2  
Filter sterilize (following filter sterilization pH to rises to 7.4)  
Keep at 4°C.

##### STO CELL MEDIUM

- 25 83.0 ml DMEM  
15.0 ml 15% fetal bovine serum (FBS); batch tested before use  
1.0 ml Pen/Strep 1:100  
1.0 ml Glutamine 1:100 (if needed) (see note below)  
30 Filter sterilize and keep at 4°C.

Note: Replenish complete medium (DMEM medium) (STO or ES) with glutamine.

- \*This step is only required if medium is older than 1 week - 10 days, as the glutamine breaks down after this  
35 time.

##### ES CELL MEDIUM WITH OR WITHOUT LIF

up to 100.0 ml DMEM

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- 15.0 ml 15% FBS (batch tested before use;  
see below)  
1.0 ml (from 0.01M stock)  $\beta$ -mercaptoethanol (0.1 mM  
final concentration)
- 5 1.0 ml Pen Strep. 1:100  
0 - 1.0 ml Glutamine 1:100 (if needed)  
1.0 ml Nystatin 1:100  
0 - 2.5 ml Recombinant murine LIF (from  $4 \times 10^4$   
U/ml; 1000U/ml stock); activity-tested using LIF Assay
- 10 (see below)  
0.4 ml Gentamicin  
1.0 ml Nucleotides  
1.0 ml Non-essential amino acids

- PENICILLIN/STREPTOMYCIN ANTIBIOTIC SOLUTION (1:100)  
15 - Commonwealth Serum Laboratories, Australia;  
Catalogue No. 05081901

Penicillin G - 5000 U/ml  
Streptomycin Sulphate - 5000  $\mu$ g/ml.

## MITOMYCIN-C SOLUTION

- 20 2.0 mg Mitomycin-C (Sigma Chemical Co. ("Sigma"));  
Catalogue No. M0503)  
200.0 ml STO Cell Medium

Filter sterilize, divide into 20x 10 ml aliquot's and  
store at -20°C.

- 25 PHOSPHATE BUFFERED SALINE (PBS)

For 100 ml Milli-Q Water:  
( $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  - containing) ( $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  - free)

- |    |  |        |       |
|----|--|--------|-------|
|    | NaCl   | 0.89   | 0.80  |
|    | KCl  | 0.02   | 0.02  |
| 30 | $\text{KH}_2\text{PO}_4$                             | 0.02   | 0.02  |
|    | $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ | 0.289  | 1.115 |
|    | $\text{CaCl}_2 - 2\text{H}_2\text{O}$                | .014   | -     |
|    | $\text{MgCl}_2 - 6\text{H}_2\text{O}$                | 0.01   | -     |
|    | Na pyruvate  | 0.0036 | -     |
| 35 | D-glucose  | 0.1 g  | -     |

Adjust to pH 7.4 and filter sterilize  
( $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  - free PBS is purchased from ICN Cell  
Biology and Tissue Culture, Cat. No. 18-604-54)

TRYPSIN/VERSENE (TV) WORKING SOLUTION (TV x 1)

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In PBS ( $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  - free):  
0.25% (w/v) trypsin (lyophilized)  
0.04% (w/v) EDTA or EGTA

or:

- 5 To 1 liter of milli-Q water add the following:

	Trypsin powder (Porcine, Difco)	2.5 g
	EDTA or EGTA	0.4 g
	NaCl	7.0 g
	$\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$	0.3 g
10	$\text{KH}_2\text{PO}_4$	0.24 g
	KCl	0.37 g
	D-Glucose	1.0 g
	Tris	3.0 g
	Phenol red	1.0 ml

- 15 Adjust to pH 7.6, filter sterilize, aliquot and store frozen.

EGTA: Ethylene-glycol-bis( $\beta$ -amino-ethyl ether) $\text{N},\text{N},\text{N}',\text{N}'$ -tetra-acetic acid [Ethylene-bis(oxy-ethylenenitrilo)]tetraacetic acid

- 20 EDTA: Ethylenediaminetetraacetic Acid

Use either EDTA or EGTA. EGTA is preferred as it is less damaging to the ES/PGC cells.

#### GELATIN WORKING SOLUTION

0.1% gelatin in Milli-Q Water

- 25 Dissolve gelatin by heating to 60°C.  
Filter sterilize when still warm.

To gelatinize tissue culture plates:

1. Cover dish with solution, leave 30 minutes
2. Aspirate gelatin and let dish air-dry.

#### 30 NUCLEOSIDE STOCK SOLUTION

	Milli-Q Water	100 ml
	Adenosine (Sigma)	80 mg
	Guanosine (Sigma)	85 mg
	Cytidine (Sigma)	73 mg
35	Uridine (Sigma)	73 mg
	Thymidine (Sigma)	24 mg

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1. Dissolve by warming to 37°C.
2. Filter sterilize and aliquot while warm.
3. Store at 4°C or -20°C.
4. Thawing of nucleotides for use in ES cell media
  - 5 (a) nucleotides come out of solution upon thawing;
  - (b) Warm to 37°C to resolubilize before use.

## NON-ESSENTIAL AMINO ACIDS (1:100)

- Commonwealth Serum Laboratories; Catalogue No. 09751301

- 10 100x concentrate for minimum essential medium (Eagle):  
(1.0 ml is added to 100 ml ES Cell Medium)

mg/10 ml milli-Q H<sub>2</sub>O

	Glycine	7.5
	L-Alanine	8.9
15	L-Asparagine · H <sub>2</sub> O	15.0
	L-Aspartic Acid	13.3
	L-Glutamic Acid	14.7
	L-Proline	11.5
	L-Serine	10.5

## 20 WHITTEN'S CULTURE MEDIUM

	KCl	0.0356
	KH <sub>2</sub> PO <sub>4</sub>	0.0162
	MgSO <sub>4</sub> · 7H <sub>2</sub> O	0.0294
	NaCl	0.4
25	NaHCO <sub>3</sub>	0.2106
	Glucose	0.1
	Na Pyruvate	0.0036
	Ca Lactate 5H <sub>2</sub> O	0.0527
	Na Lactate	0.2416 ml
30	Milli-Q-H <sub>2</sub> O	100 ml

The solution is adjusted to a final milliosmolarity of 250-280 by addition of H<sub>2</sub>O or NaCl.

Filter sterilize and store at 4°C for two weeks.

Working solution:

- 35 10 ml Whitten's medium



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1.5g  
Diagnostic division,  
Code No. 81-001-4)

BSA fraction V (Miles Pentex,  
Kankakee, Il., USA;

Filter Sterilize and equilibrate in 5%O<sub>2</sub>:5%CO<sub>2</sub>:90% N<sub>2</sub> at  
5 39.5°C, 95% humidity.

#### FBS BATCH TRIALS

Batches of FBS vary in the ability to support growth of ES cells, and in the ability to maintain the undifferentiated state of such cells. The following  
10 procedure is used to identify suitable batches of FBS.  
Use ES cells from between 2 & 20 passages:

##### Day 1

Split ES colonies and plate into dishes  
without feeder cells but with LIF.  
15 Incubate for 3 days.

##### Day 4

Trypsinise to detach colonies and cells.  
Count cells and dispense into  
gelatinized 6cm dishes  
containing ES Cell Medium and  
20 LIF (no serum added) as follows:

Dish Number		No. Cells	Batch FBS Control Serum (Batch Tested)		
Non-Inactivated Serum			A	B	
25	1	250	5 ml	-	-
	3	250	-	5 ml	-
	5	250	-	-	5 ml
	7	2000	5 ml	-	-
	9	2000	-	5 ml	-
30	11	2000	-	-	5 ml
Inactivated Serum, as control (56°C for 15 min.)					
35	13	250	5 ml	-	-
	15	250	-	5 ml	-
	17	250	-	-	5 ml
	19	2000	5 ml	-	-
	21	2000	-	5 ml	-
	23	2000	-	-	5 ml

There are duplicate plates for each treatment.

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Incubate low density dishes for 5 days  
Incubate high density dishes for 3 days

Day 7 Fix high density cells and stain with hematoxylin.

Day 9 Fix low density cells and stain for alkaline  
5 phosphatase.

#### LIF ASSAY

This procedure is used to assay the potency of  
Leukaemic Inhibitory Factor (LIF).

Day 1 Split one 10 cm dish of confluent STO cells  
10 into five dishes. Incubate for 2 - 3 days in  
STO medium.

Day 3/4 When cells are confluent, replace medium with  
DMEM + 10% FBS. Incubate for 3 days.

Day 6/7 Collect conditioned medium (CM) and store at  
15 4°C.

\*Prepare low density ES cell cultures as described above.

Dish	No. Cells	C.M.	Medium	1000 U/ml LIF	Medium w/o LIF	Presumed LIF Content
20 1,2,3	250	0.1 ml	4.9 ml	-	-	200 U/ml
4,5,6	250	0.25 ml	4.75 ml	-	-	500 U/ml
7,8,9	250	0.5 ml	4.5 ml	-	-	1000 U/ml
10,11,12	250	1.0 ml	4.0 ml	-	-	2000 U/ml
13,14,15	250	-	-	5 ml	-	
25 16,17,18	250	-	-	-	5 ml	

There are triplicate plates for each treatment.

Fix and stain for alkaline phosphatase.

#### Preparation of Fibroblast Feeder Cell Layers

Embryonic pluripotential cells are cultured in  
30 vitro on a layer of fetal fibroblast cells. The  
fibroblast cells provide a wide range of factors  
necessary for the growth of pluripotential embryonic

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cells (e.g. growth factors, cytokines, factors that are essential for maintenance of ES cell pluripotency).

ISOLATION OF PORCINE FETAL FIBROBLASTS:

1. Remove developing porcine fetuses (preferably  
5 between days 16-30 of development) from uterus by aseptic dissection.
2. Remove skin layer from fetus.
3. Dissect out soft tissue avoiding developing viscera.  
The white (fibroblast containing) tissue is found  
10 just under the skin layer.
4. Wash dissected tissue in PBS ( $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  free).  
Centrifuge at 1000 rpm for 5 min.
5. Remove supernatant.
6. Incubate tissue in Trypsin/Versene Working Solution  
15 for 20 min.
7. Dissociate cells by vigorously pipetting.  
Centrifuge at 1000 rpm for 5 min.
8. Remove supernatant.
9. Resuspend cells in STO Cell Medium. Allow large  
20 cell-clumps to settle.
10. Plate out cells within supernatant (i.e., large cell  
clumps are not included) onto gelatinized tissue  
culture plates. Incubate cells in an atmosphere of  
5%  $\text{CO}_2$ , 95% air (37.5°C, 95% humidity) until a  
25 confluent layer of fibroblast cells appears (~4-5  
days).
11. Passage of cells may be continued to increase cell  
numbers, or cells may be frozen or inactivated for  
further use.

30 CULTURE OF FETAL FIBROBLAST FEEDER LAYERS FROM FROZEN STOCKS:

Several different types of mouse feeder (STO cells) and porcine and bovine fetal fibroblasts can be used to form feeder layers. These include:

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- (1) Bradley/Baylor mouse STO feeder cells that have been modified to express human LIF (gift from Allan Bradley, Institute for Molecular Genetics, Baylor College of Medicine, Texas Medical Center, Houston, Texas, USA)
- (2) Robertson/Columbia mouse STO feeder cells that have been modified to express murine LIF (gift from Elizabeth Robertson, Columbia University, New York, USA)
- (3) Several porcine fetal fibroblast lines
- (4) Several bovine fetal fibroblast lines

(the fibroblast lines of (3) and (4) were derived by the present inventors using the procedures described above)

15 The procedure for producing feeder layers is as follows:

1. Rinse one 10 cm tissue culture (tissue cure) dish with gelatin/Milli-Q water solution for 30 min. Aspirate gelatin solution and let dish air-dry.
3. Add 10 ml of STO cell medium to 15 ml centrifuge tube.
4. Remove feeder layer cells frozen in freezing media from liquid N<sub>2</sub> container.
5. Thaw cells by warming vial in hands or in 37°C water bath.
6. Transfer STO cells to medium in centrifuge tube.
7. Spin at 1000 rpm for 5 min.
8. Resuspend cells in 10 ml medium and transfer to gelatin-treated tissue culture dish.
9. Incubate at 37°C for 3 days.

30 SPLITTING OF FEEDER LAYER STO CELL/FETAL FIBROBLASTS:

This procedure is used to expand the number of cells from a single confluent plate/dish; cells are detached from the confluent plate and transferred to fresh plates at sub-confluent densities.

1. Gelatinize five 10 cm tissue culture dishes.
2. Examine incubated STO cells under microscope and check for confluence.

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3. If STO feeder monolayer is confluent (cells cover bottom of dish, or nearly so), wash gently with PBS ( $\text{Ca}^{++}$  and  $\text{Mg}^{++}$  - free) for 1 min.
4. Aspirate PBS and add 1 ml Trypsin/Versene Working  
5 Solution for 1 min (or until cells start to detach).  
Check under microscope.
5. Detach cells by vigorously pipetting, add 1.0 ml STO  
Cell medium (i.e., a ratio of 1:1 STO Cell  
medium:Trypsin/Versene Working Solution) to  
10 neutralize trypsin, and transfer to a centrifuge  
tube containing 10-15 ml STO Cell medium. Wash  
cells remaining on dish with some of STO cell medium  
from the tube. Centrifuge at 1000 rpm for 5 min.,  
aspirate supernatant, resuspend pellet in 1 ml STO  
15 Cell medium. Resuspend cells to make single cell  
suspension. Make up to 50 ml with STO Cell medium.
6. Dispense 10 ml into each of the five tissue culture  
dishes and incubate until confluent (~ 3 days).

#### INACTIVATION OF FEEDER LAYERS:

- 20 The present inventors use two alternative methods  
for inactivating feeder layers, which stops the cells  
from dividing:

##### (1) Mitomycin treatment:

1. Check dishes for confluence of STO cells/fetal  
25 fibroblasts.
2. Thaw mitomycin-C solution and use undiluted.
3. Aspirate STO cell medium from feeder cell plate.
4. Add 10 ml aliquot of mitomycin-C to plate and  
incubate at 37°C for 1-3 hours.
- 30 5. Aspirate mitomycin-C, wash cells in 1x PBS (without  
 $\text{Ca}^{++}$  or  $\text{Mg}^{++}$ ) for 1 min.
6. Aspirate PBS and add 1 ml trypsin solution for 1  
min.

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7. Detach cells by vigorously pipetting and transfer to STO cell medium in centrifuge tube.
8. Centrifuge at 1000rpm for 5 min.
9. Resuspend cell pellet in 1 ml ES Cell Medium.
- 5 10. Plate out in dishes in preparation for addition of ES cells.

(2) Gamma Irradiation:

1. Check dishes for confluence of STO cells/fetal fibroblast.
- 10 2. Trypsinise cells into single cell suspension.
3. Irradiate cells (3000 rads) in STO cell medium.
4. Centrifuge at 1000 rpm for 5 min.
5. Resuspend pellet in 1 ml ES Cell Medium.
6. Transfer cells to gelatinized tissue culture  
15 dishes with ES Cell Medium and place in incubator at 37°C until the cells adhere to the dish. NOTE: If cells are not confluent, count using hemocytometer and seed at  $5 \times 10^4$  cells in 1 ml medium per well of Nunc 4-well plate.
- 20 One 10 cm dish of inactivated cells can be split into:  
Ten 4-well plates (Nunc tissue culture plates),  
or Eight 3.5 cm tissue culture dishes, or  
Three 6 cm tissue culture dishes, or  
25 Two 20 cm tissue culture dishes.

Demonstration of Totipotency:

A. Blastocyst Injection

- The ability of embryonic cell lines to form germline chimeric animals is a conclusive test for their  
30 totipotency. This can be accomplished by blastocyst injection experiments, using techniques for various mammalian species substantially the same as those established for the mouse. See Example 14, below. See also, e.g., Bradley, Production and Analysis of Chimeric

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Mice, In: Teratocarcinomas and Embryonic Stem Cells: A Practical Approach (E.J. Robertson, ed.), IRL Press, Oxford, pp. 113-52 (1987). However, for porcine manipulations the holding pipette must be somewhat larger  
5 as porcine embryos are larger than mouse embryos.

B. Co-Culture of ES Cells/PGC's and Morula Embryos

Embryos at the morula stage of development are surgically collected from superovulated animals. For porcine embryos, for example, the zona pellucida is then  
10 disrupted using Acid Tyrodes solution and ES cells/PGC's are cultured in the presence of the zona pellucida-disrupted morulae. ES/PGC cells adhere to the exposed morula cells and, following overnight culture in Whitten's medium, the embryos are transferred to  
15 synchronized recipients. Preferably, the zona pellucida-disrupted morula is completely free of the zona pellucida. However, this need not be the case as long as the ES cells/PGC's can gain direct access to at least some of the morula cells.

20 C. Morula Injection

ES cells and PGC's can be injected into a morula embryo prior to formation of the blastocyst cavity. The technique is similar to blastocyst injection. ES cells or PGC's are drawn into an injection pipette, which is  
25 inserted beneath the zona pellucida. Then, the cells are expelled so that they are in contact with the cells of the morula embryo. The injected morula is then cultured overnight in Whitten's medium (porcine) or other appropriate medium to allow blastocyst formation.

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#### D. Nuclear Transfer and Embryo Cloning

ES cells and PGC's can be fused to enucleated zygotes that have been derived by in vitro maturation, in vitro culture, in vitro fertilization or collected surgically. Following successful fusion the embryos can be transferred to synchronized recipients. *In vitro* or *in vivo*-collected porcine oocytes, for example, are manipulated in Whitten's medium supplemented with 1.5% BSA Fraction V and 7  $\mu$ g/ml cytochalasin B (Sigma). A bevelled micropipette is used to remove the metaphase plate from the oocyte. A single ES cell or PGC (after trypsin treatment to form a single-cell suspension) is inserted through the zona using a bevelled micropipette, such that the cell comes in contact with the oocyte plasma membrane. Fusion is achieved in a 28 V/cm AC field for 5 sec. followed by an 80 V/cm DC pulse of 100  $\mu$ sec. duration. Subsequent to observed fusion, embryos are incubated at 39° C in 5% CO<sub>2</sub>, 5% O<sub>2</sub>, 90% N<sub>2</sub> in microdrops of Whitten's medium supplemented with 1.5% BSA, until transfer to a synchronized recipient.

#### EXAMPLE 11

##### Murine ES Cell Culture

ES cells are able to differentiate spontaneously into many different cell types, and culture conditions which prevent this differentiation are critical for the continuous passage of these cells in an undifferentiated form, capable of contribution to chimeric mice.

#### I. CULTURE CONDITIONS

ES cells are grown in polystyrene cell culture dishes treated with 0.1% gelatin (made up in PBS or Milli-Q water) for 10 minutes. A feeder layer of mitotically inactivated fibroblasts provides a source of cytokines. The fibroblasts are either primary mouse embryo fibroblasts (PMEFs), or STO fibroblasts, an



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immortal line. The medium used is DMEM supplemented with glucose, amino acids and nucleosides. Robertson, Embryo-Derived Stem Cell Lines. In: Teratocarcinomas and Embryonic Stem Cells: A Practical Approach (E.J. Robertson, ed.), IRL Press, Oxford (1987). To this medium is added LIF (final concentration of  $10^3$ U/ml Esgro, AMRAD). FBS is added to 15%. The batch of FBS is chosen on the basis of its ability to support ES cell growth with low levels of differentiation (i.e., only rare individual cells undergo differentiation. The ES cells are grown in an atmosphere of 5-10% CO<sub>2</sub>, at 37°C

## II. ROUTINE PASSAGE

ES cells must be passaged frequently to prevent the colonies from growing too large and differentiating. This is achieved by splitting the cells at a ratio of 1:10 to 1:40, every two to four days.

## EXAMPLE 12

### Genetic Manipulation of Cells

The general procedures set out in this Example provide guidelines that are readily adaptable to individual experimental situations that might employ, for example, different cell lines or equipment supplied by different manufacturers. This Example also provides specific procedures used and results obtained in generating a set of mouse ES cell lines in which the  $\alpha$  1-3 galactosyltransferase gene was disrupted by homologous recombination. The general procedures provided in this Example are adapted for mouse ES cells. However, the procedures are substantially similar for porcine ES cells.

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I. INTRODUCTION OF DNA INTO ES CELLS BY ELECTROPORATION

A. Coat required number of plates with 0.1% gelatin (in PBS or Milli-Q water). (Usually 2 X 6 well plates and 8 well plate)

5 B. Thaw  $10^7$  embryonic fibroblasts into DMEES (equivalent to ES Cell Medium); inactivate by irradiating at 3000 Rad.

C. Count irradiated cells, spin down and resuspend in DMEES to  $10^6$  cells/ml.

10 D. Aspirate gelatin from plates and plate cells at:  $7 \times 10^5$  cells/well (6 well plate) in 2.5ml medium;  $7 \times 10^4$  cells/well (24 well plate) in 1 ml medium. Incubate at  $37^\circ\text{C}$ , 5-10%  $\text{CO}_2$  for 3 - 4 hr.

E. Wash ES cells in 5 ml (250 ml flask) PBS-EGTA  
15 and let sit at room temperature for 4 min.

F. Remove PBS, add 5 ml trypsin (CSL) and leave at room temperature for 2 - 4 min. Wash down cells, add 10 ml DMEES and count. Approximately  $5 \times 10^6$  to  $2 \times 10^7$  ES cells are needed for experiments.

20 G. Centrifuge cells and resuspend in 10 ml PBS. Centrifuge again and resuspend in  $540 \mu\text{l}$  PBS. Dilute  $50 \mu\text{l}$  into 10 ml DMEES and culture to determine plating efficiency.

H. Add 5 - 10  $\mu\text{g}$  DNA to cells in 10  $\mu\text{l}$  PBS (total  
25 volume,  $500 \mu\text{l}$ ) and transfer to sterile electroporation cuvette (e.g. Biorad).

I. Electroporate at 0.22 kV, 500  $\mu\text{FD}$  (time constant should be  $\sim 8.4$ ). This is achieved using a Biorad Gene Pulser unit (Biorad Catalogue No. 1652078)  
30 with capacitance extender (Biorad Catalogue No. 1652087), or similar device.

J. Resuspend in 10 ml DMEES with constant pipetting to break up clumps of DNA from lysed cells.

K. Centrifuge cells and resuspend in 5ml DMEES.

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L. Take 50  $\mu$ l, add 50  $\mu$ l trypan blue solution and count for viability.

M. Culture by dilution plating to determine plating efficiency.

## 5 II. SELECTION CONDITIONS

ES cells that do not express a neomycin resistance gene are selectively killed by treatment with G418 at 200-500  $\mu$ g per ml of medium. Antibiotic- containing medium is changed daily. A population of cells that has  
10 not been electroporated also is treated in order to see how genuinely sensitive cells respond to the G418 treatment. After 6 to 10 days, cells resistant to the antibiotic will be evident as healthy colonies. These cells will have been transformed by the targeting  
15 construct and can be screened for homologous recombination (i.e., screened for gene targeting versus random integration).

Resistant colonies are picked from the selection dish with a mouth pipette and dispersed into a single  
20 cell suspension. Half of these cells are frozen away while the other half is expanded and used to determine whether or not homologous recombination has occurred. If the colonies are small, it is sometimes preferable to expand the whole colony in a 24 well dish, and then to  
25 freeze half while further expanding the other half for genetic analysis.

## III. PICKING ES CELL COLONIES FOR GENETIC ANALYSIS AFTER SELECTION

### A. Method 1: Freezing Half Colonies

- 30 1. The day before colony picking:
- a) Coat required number of plates with 0.1% gelatin (in PBS). Two plates per 24 colonies to be picked: one plate is for freezing and one plate

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is for clone expansion. Start with 20 X 24 well plates.

- b) Count irradiated fibroblasts, spin down and resuspend in DMEES.
- 5 c) Aspirate gelatin from 10 plates and plate  $\sim 10^5$  (can use as few as  $5 \times 10^4$ ) cells/well in 1ml DMEES. Incubate at 37°C, 10% CO<sub>2</sub> overnight (or a minimum of 1 h).
- 10 d) Aspirate the gelatin from the other 10 plates.
- 2. On the day of colony picking:
  - a) 15 Change medium on ES cells before and regularly during picking (to remove floating cells).
  - b) 20 Pull plugged pasteur pipettes. Use a fresh pipette after each 24 colonies. The desired tip is about half a colony in diameter, with the constriction over 1-2cm. The tip should be perpendicular and neat. Note: after drawing the pipette, rub the glass at the desired break point with freshly drawn glass, then bend.)
  - c) 25 Label multi-tip reservoirs for:
    - 1 PBS-EGTA
    - 2 Trypsin-Versene
    - 3 DMEES
    - 30 4 2 X Freezing mix(20% DMSO in FCS)
  - d) Using multipipettor, dispense 50  $\mu$ l PBS-EGTA into 24 wells of 96 well plate.
  - e) 35 At microscope: Connect finely drawn pasteur pipette to mouth pipette tube. Dislodge colony from plate and transfer (in minimum volume) to one well of a 96 well plate. Expel contents of pipette; the bubbles serve as a location guide. Pick 24 colonies or as many as possible in 40 <10-15 min (preferably a multiple of 6).

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- 5 f) Back in hood: Add 100  $\mu$ l trypsin to each well using multipipettor) and leave at RT for 2 min.
- g) Pipette up and down 10- 15X to disperse cells, then add 100  $\mu$ l DMEES. (This should be done within 4-6 min after trypsin addition).
- 10 i) Divide cell suspension between freezing and expansion plates using 12 channel pipette with every second tip fitted. Transfer 125  $\mu$ l to gelatinized 24 well plate (to freeze); the remaining ~125  $\mu$ l is transferred to a 24 well plate with feeder layer (for DNA). The plates are labelled and carefully aligned to ensure that one clone goes into the same well of each tray.
- 15 j) Add 125  $\mu$ l 2 X freeze mix to each well on freezing plate, mix well by swirling.
- 20 k) Seal in ziplock bag or plastic wrap and place in -70°C freezer in an equilibrated styrofoam box. Interleave the plates with styrofoam sheet.
- 25 l) Incubate expansion plates until there are sufficient cells for genotype analysis.
- 30 A. Method 2: Freezing after expansion to 24 wells.
1. The day before colony picking:
- 35 a) Coat required number of plates with 0.1% gelatin (in PBS). Start with 10 X 24 well plates.
- b) Count irradiated fibroblasts, spin down and resuspend in DMEES.
- 40 c) Aspirate gelatin from the plates and plate  $\sim 10^5$  cells/well in 1ml DMEES. Incubate at 37°C, 10% CO<sub>2</sub> overnight (or a minimum of 1 h).

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## 2. On the day of colony picking:

- 5 a) Pick colonies as described for half colonies (method 1, above) but instead of dividing the cell suspension between freezing and expansion plates, the entire cell suspension goes into the expansion plate.
- 10 b) After 3-4 days (with daily medium changes) the cells will have grown sufficiently to be frozen. Working one plate at a time (with practice two can be handled), aspirate medium from each well. Flood with PBS/EGTA for 4 minutes. Meanwhile, set up pipette tips to fit alternate channels of a twelve channel multipipettor. Aspirate PBS.
- 15
- 20 c) Add 100  $\mu$ l trypsin (using multipipettor and alternate channels) and leave at room temp. for 2 min.
- 25 d) Pipette up and down 10- 15X to disperse cells of first row, change tips, then add 100  $\mu$ l DMEES. Repeat for each row. (This should be done within 6 min of trypsin addition).
- 30 e) Using 12 channel pipette with every second tip fitted, transfer 125  $\mu$ l to gelatinized 24 well plate (to freeze). The remaining cells will be expanded for DNA. It is crucial that the plates are labelled and carefully aligned to ensure that the freezing tray matches the expansion tray.
- 35 f) Add 125  $\mu$ l 2 X freeze mix to each well on freezing plate; mix well by swirling.
- 40 g) Seal in ziplock bag or plastic wrap and place in -70°C freezer in an equilibrated styrofoam box. Interleave plates with styrofoam sheets.
- 45 h) Add 1ml of DMEES to the expansion tray. (There will be sufficient feeder cells to give good plating

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efficiency). Incubate for 3-4 days until there are sufficient cells for genotype analysis.

#### IV. THAWING OF ES CELL CLONES FROZEN IN 24-WELL PLATES

- 5 Cells that have been identified to have the desired genetic alteration are recovered from a duplicate plate frozen at  $-70^{\circ}\text{C}$ . The plate is taken to the laminar flow hood and removed from the plastic bag. Each well is filled with warm medium, and feeder cells are added to
- 10 the well(s) of interest. The plate is placed in a  $37^{\circ}\text{C}$  incubator for 60 min., then the medium is replaced. Colonies will appear after two or three days. These colonies are expanded for establishment of new frozen stocks, and tested for 1) karyotype analysis; 2)
- 15 confirmation of the desired genetic alteration; 3) mycoplasma infection; and 4) ability to form chimeras.

#### EXAMPLE 13

##### Production Of Mouse ES Cell Knockouts Using The pNEO $\alpha$ GT10.8B Construct

#### 20 I. TRANSFORMATION

- A total of  $1 \times 10^7$  E14 ES cells was electroporated with  $5\mu\text{l}$  of  $1\mu\text{g}/\mu\text{l}$  pNeo $\alpha$ GT10.8B DNA (linearized by XhoI digestion) (see Example 9 and Figure 17). Electroporation was carried out in  $600\mu\text{l}$  in a wide cuvette at  $25\mu\text{F}$ , 350V
- 25 for 0.5msec. Cells were recovered in 6ml ES complete medium and plated into 6 x 100mm petri dishes, each containing a feeder layer of Neo<sup>R</sup> STO cells.

- Cells were cultured in ES complete medium for 3 days and then medium containing 200-350 $\mu\text{g}/\text{ml}$  G418 was
- 30 substituted. This medium was changed every second day. After 9 days, individual Neo<sup>R</sup> colonies were sufficiently large to be identified and recovered. Colonies were picked in  $20\mu\text{l}$  PBS and  $20\mu\text{l}$  of trypsin solution were added. Forty  $\mu\text{l}$  of 60% BRL conditioned medium in ES

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complete medium were then added. Aliquots of 40 $\mu$ l were transferred to single wells of each of two 24-well plates. One plate contained a feeder layer of STO cells in 100 $\mu$ l ES complete medium. 140 $\mu$ l of 2x DMSO freezing mix was added to this plate, which was stored at -80°C. Each of the wells of the second 24-well plate contained 1ml of 60% BRL conditioned medium in ES complete medium. This plate was incubated at 37°C until the colonies were confluent.

10 II. CONFIRMATION OF HOMOLOGOUS RECOMBINATION

Medium was aspirated off confluent colonies and 400 $\mu$ l lysis buffer (10mM Tris pH 7.8, 100mM NaCl, 1mM EDTA, 1% SDS, and 500 $\mu$ g/ml Proteinase K) added. The cells were lysed at 37°C overnight, extracted with 400 $\mu$ l 1:1 phenol/chloroform and transferred to Eppendorf tubes containing 1ml 95% ethanol and 0.2M NaAc. DNA was pelleted by centrifuging at 13,000 rpm in an Eppendorf centrifuge, the pellet washed twice with 80% ethanol and redissolved in 30 $\mu$ l water.

20 Southern analysis (see, e.g., Sambrook et al., supra) was used to identify ES cell clones where homologous recombination had occurred at the 3' end of the construct. Aliquots of 15 $\mu$ l of DNA were digested with 20 units of the restriction enzyme BglII according to the manufacturer's recommendations. After incubation at 37°C overnight, the DNA was electrophoresed through a 0.8% agarose gel (in a Tris acetate, EDTA buffer) at 1-2V/cm overnight, using 750ng of HindIII-digested lambda DNA as markers. The DNA was transferred to a Zetaprobe nylon membrane using a Hybaid vacublotter at a vacuum of 80cm Hg for 1 hour.

The membrane was prehybridised in a Hybaid hybridization bottle in 10ml of the following hybridization mix for 3 hours at 65°C:



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5                   0.25M Na<sub>2</sub>HPO<sub>4</sub> pH 7.2  
                  7% SDS  
                  1mM EDTA  
                  100µg/ml salmon sperm DNA  
                  10% PEG

Radioactively labeled probe DNA was prepared using a BRESATEC gigaprime oligo labeling kit (Cat. No. GPK-1) according to the manufacturer's recommendations. Approximately 50ng of a 0.7kb EcoRI/XmnI DNA fragment from beyond the 3' terminus of the construct pNeoαGT10.8B (see Example 9 and Figure 17) were labeled with <sup>32</sup>P-dATP to a specific activity of 5x10<sup>8</sup> cpm/µg. The denatured probe was added to the prehybridising membrane in the Hybaid bottle and incubated overnight at 65°C.

15       The membrane was removed from the Hybaid bottle, rinsed with 0.5xSSC, 0.1% SDS prewarmed to 65°C, and then washed 2-3 times with 0.1xSSC, 0.1% SDS at 65°C for 30 min each wash. Excess moisture was then blotted from the membrane, the membrane wrapped in plastic wrap and

20       exposed to a phospho-imager screen for 16 hours up to 3 days. The image was visualized on an Imagequant phospho-imager.

Results are shown in Figure 18, which is a Southern blot of DNA from 15 ES cell lines probed with the

25       diagnostic 0.7kb EcoRI/XmnI DNA fragment described above and in Example 9. The 6.4kb band, diagnostic for a homologous recombination event in the α 1-3 galactosyltransferase gene (α 1-3 Gal T) (see Example 9), is seen in 6 of the 15 ES cell lines examined. All of

30       the 6 knockout cell lines appeared to be heterozygous for the inactivated allele since the 8.3kb band, diagnostic for the uninterrupted α-1,3-Gal T gene (see Example 9), was also present in all six lanes.

Two cell lines, designated hereinafter "8D1" and

35       "7C2," were chosen for further analysis. Cell lines 8D1 and 7C2 were identified by Southern analysis to contain

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an  $\alpha$ -1,3-Gal T allele where homologous recombination had occurred at the 3' boundary of the construct.

Long range PCR was then used to determine whether or not homologous recombination had occurred at the 5' boundary of the construct within these cell lines. Two sets of primers were used in separate PCR experiments:

1) Wild-type primers:-

MGT-KOex8F and MGT-KOR1 span the intron between exons 8 & 9, and amplify a 5.5 kb fragment from the wild-type  $\alpha$ -1,3-GalT gene (Figure 19)

SEQUENCES:

MGT-KOex8F

5'TGCTGGAAAAGTACTACGCCACACAGAAACTCA-3'

(SEQ ID NO: 14)

(Nucleotides 1014-1046 in Figure 4)

MGT-KOR1

5'AGCCAGAGTAATAGTGTCAAGTTTCCATCACAA-3'

(SEQ ID NO: 15)

(Nucleotides 1779-1811 in Figure 4)

2) Knockout primers:-

MGT-KOex8F and MGT-KONeoR span exon 8 to the Neo<sup>R</sup> gene cassette in the "knock-out" allele and amplify a 5.5 kb fragment from the knocked out allele (Figure 19)

SEQUENCE:

MGT-KONeoR

5'-GCCACACGCGTCACCTTAATATGCCAAGTGGAC-3'

(SEQ ID NO: 16)

(Nucleotides 323-355; Figure 16)

Each reaction contained ~100 ng genomic DNA as template in a reaction volume of 50  $\mu$ l and contained 25mM Tris HCl (pH9.1), 16mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 250  $\mu$ M dNTPs, 3.5 mM MgCl<sub>2</sub>, 100 ng each primer, 2 units Taq polymerase and 0.025 units Pfu polymerase. The reactions were heated at 94°C for 1 min, then 45 cycles of 94°C for 15 sec, 68°C

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for 6 min, followed by a single step of 72°C for 10 min. Genomic DNAs from putative "knock-out" ES cell lines from CBA/C mice (homozygous for the wild-type  $\alpha$ -1,3-Gal T allele) were amplified in separate reactions using each set of primers. A 10 $\mu$ l aliquot of each PCR was analyzed by Southern blotting (Sambrook et al., 1989).

The results are illustrated in Figure 20:

Knockout primers:-

10 A 5.5 kb fragment that hybridized to the 1.3 kb Neo<sup>R</sup> gene cassette (Figure 16) was generated from 7C2 DNA (Figure 20; lane 4) and 8D1 DNA (not shown). This band was not generated from CBA/cDNA (Figure 20; lane 3).

Wild-type primers:-

15 A 5.5 kb fragment that hybridized to the  $\alpha$ -1,3-Gal T gene probe (isolated by Sal I digestion of p $\alpha$ GT-S4.0) was generated from 7C2 and CBA/cDNA's (Figure 20; lanes 1 and 2 respectively) and 8D1 DNA (not shown). This product did not hybridize to the Neo<sup>R</sup> gene  
20 probe.

These results demonstrate that homologous recombination had occurred at the 5' boundary of the construct in cell lines 8D1 & 7C2.

#### EXAMPLE 14

##### 25 Generation of Animals Carrying an ES Cell Genome

The procedures provided in this Example are adapted for mouse ES cells. However, the general strategy is substantially the same for porcine ES cells and PGC's.

#### I. PREPARATION OF ES CELLS FOR INJECTION

30 ES cells are split into wells of a 24-well dish at cell densities of 1:2, 1:4, 1:8 and 1:16, relative to the initial density, two and three days before injection.

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The most vigorous and least differentiated cultures are chosen on the basis of morphology.

## II. EMBRYO INJECTION AND PRODUCTION OF CHIMERIC MICE

Mouse embryos are collected from either  
5 superovulated or naturally mated female mice, approximately  
3.5 days after mating. After overnight culture in M16  
medium (Bradley, Production and Analysis of Chimaeras. In  
Teratocarcinomas and Embryonic Stem Cells a Practical  
Approach (E.J. Robertson, ed.) IRL Press, Oxford, pp. 113-  
10 52 (1987)), those that have cavitated to form blastocysts  
are microinjected with about 12 to 20 ES cells. This  
microsurgical procedure is performed with instruments drawn  
from capillary glass, and injection is controlled with  
micrometer syringe-based hydraulic devices. A differential  
15 interference contrast-equipped inverted microscope is used  
to view the procedure.

After injection, blastocysts are transferred to  
the uterus of pseudopregnant female mice. Chimeric mice  
are identified by coat color contribution by the ES cells.  
20 Chimaeric mice show agouti coat colour derived from the  
host blastocyst, and chinchilla contributed by the ES  
cells.

Chimeric mice were generated from ES cells carrying  
the interrupted a-1,3-Gal T allele (including 8D1, 7C2  
25 cells) by injection into C57B1/6J x CBA F2 blastocysts. The  
ability of individual chimaeric mice to transmit the ES  
cell characteristics through the germ-line was estimated by  
glucose phosphate isomerase (Gpi) analysis of sperm  
(Bradley, supra, (1987)); Mann et al., J. Reprod & Fert.  
30 99, 505-512 (1993). Glucose phosphate isomerase catalyses  
the interconversion of glucose-6-phosphate to fructose-6-  
phosphate. Mice have a single structural Gpi locus with  
two main alleles Gpi 1A and Gpi 1B. Gpi 1A codes for  
protein which appears as a slow cathodically migrating band

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during electrophoresis and occurs in strains such as BALB/c and C129. (The ES cells used here were derived from strain 129 mice). Gpi 1B determines an enzyme that moves faster than Gpi 1A and occurs in the wild and in strains such as  
5 C57 and CBA (used here to derive host blastocysts).

Heterozygotes have the two parental bands plus an intermediate band which indicates the dimeric structure of the enzyme. Multiple electrophoretic forms occasionally observed are due to oxidation of sulfhydryl groups and not  
10 due to tissue-specific expression. In chimaeric mice, the ratio of Gpi 1A (strain 129-derived) to Gpi 1B (derived from the host blastocyst) indicates the proportion of cells with the ES cell genotype within different tissues. The appearance of Gpi 1A (derived from the ES cells) in the  
15 sperm suggests that the mouse is able to transmit the ES cell genotype through the germ-line.

### III. GENERATION OF MICE HOMOZYGOUS FOR THE GENETIC CHANGE INTRODUCED INTO THE ES CELLS.

Chimaeric mice with sperm derived from ES cells were  
20 mated to BALB/c mice. Offspring with the 129/Ola X BALB/c genotype (i.e. heterozygous for the ES cell genotype) are grey. Half of these grey mice were expected to carry the interrupted allele. Mice heterozygous for the interrupted allele were identified by PCR analysis of genomic DNA  
25 obtained from blood.

To generate mice homozygous for the inactivated  $\alpha$ -1,3-Gal T gene, the heterozygous mice were mated to each other. One quarter of the offspring were expected to be homozygous for the interrupted gene. Homozygotes were identified by  
30 PCR analysis of genomic DNA obtained from blood. The PCR strategy was based on the insertion of a Neo<sup>R</sup> gene in the Sal I site of exon 9 of the  $\alpha$ -1,3-Gal T gene (Figure 13).  
Wild-type primers:-

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E9F: 5'TCAGCATGATGCGCATGAAGAC 3'

(SEQ ID NO: 17)

(homologous to sequence about 40 to 60 bp 5' to the  
Sal I site of exon 9, corresponding to nucleotides 1257-  
5 1278; Figure 4)

E9R2: 5'TGGCCGCGTGGTAGTAAAAA 3'

(SEQ ID NO: 18)

(homologous to a region about 175 to 195 bp 3' to the  
Sal I site of exon 9, corresponding to nucleotides 1511-  
10 1492; Figure 4)

The expected fragment size generated from the wild-  
type allele is 255 bp (Figure 21). These primers also can  
potentially generate a 1596 bp PCR fragment from the  
interrupted allele. In practice this fragment was not  
15 generated when both the wild-type and interrupted alleles  
were present, probably because the smaller 255 bp product  
is amplified preferentially.

Knock-out primers:-

NeoF1: 5' TCTTGACGAGTTCTTCTGAG 3'

20 (SEQ ID NO: 19)

(corresponding to nucleotides 1170-1189; Figure 16)

E9R2: (the same primer described above to detect the  
wild-type allele)

The expected fragment size is 364 bp (Figure 21).

25 Mice were grown to weaning age and bled from the  
tail. Sodium Heparin was added to about 10 U/ml. PCR  
amplification was conducted on 1  $\mu$ l of heparinised blood  
( $\sim 10^4$  nucleated cells) in a 50  $\mu$ l reaction volume containing  
100 mM Tris-Acetate pH 8.8, 3.5 mM  $MgCl_2$ , 0.2mM dNTPs, and  
30 2 units Tth DNA polymerase. Each reaction contained both  
the wild-type and knock-out primers at a concentration of  
2ng/ $\mu$ l for each primer. To ensure that Tth polymerase was

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not inhibited by heparinized blood, each reaction was performed in duplicate.

One of the reactions was spiked with two DNA samples:

i) 10 fg (~600 molecules) of linearized KO plasmid  
5 pNeoαGT10.8B.

ii) 1 fg (~1000 molecules) of a 983 bp RT-PCR product that includes Exon 9.

The other reaction was not spiked. Thus, two separate PCR reactions were set up for each blood sample. In addition,  
10 control PCR reactions with no genomic DNA template and with or without spikes were conducted. Each reaction mix was heated at 94°C for 3 min., then incubated for 40 cycles at 94°C for 40 sec., 53°C for 40 sec., and 72°C for 40 sec. Aliquots of 5 µl of each reaction mix were electrophoresed  
15 on a 3% agarose gel, and DNA fragments were visualized on a UV light box after staining with ethidium bromide. HpaII-digested pUC19 plasmid DNA was used for markers.

Results of the PCR analysis for three mice, and a "no DNA" control, are shown in Figure 22. For mouse #42, the  
20 KO primers generated a 364 bp band in the + spike reaction only. The wild-type primers generated a 255 bp band in the + spike and - spike reactions. These results demonstrate that mouse #42 is homozygous for the wild-type allele. For mouse #43, the wild-type primers generated a 255 bp band in  
25 the + spike reaction only. The KO primers generated a 364 bp band in the + spike and - spike reactions. These results demonstrate that mouse #43 is homozygous for the interrupted allele. For mouse #44, the KO primers generated a 364 bp band in the + spike and - spike  
30 reactions. The wild-type primers generated a 255 bp band in the + spike and - spike reactions. These results demonstrate that mouse #44 is heterozygous for the interrupted allele. In the control PCR reactions, no product was evident when template was not included. PCR  
35 products of 364 bp and 255 bp were evident when

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pNeo $\alpha$ GT10.8B and Exon 9 RT-PCR DNA were the only templates included in the control reactions.

## EXAMPLE 15

Characterization of Homozygous Knockout Mice

## 5 I. ABSENCE OF Gal T mRNA IN Gal T KNOCKOUT MICE

A. RNA Isolation

Total RNA was extracted using the RNazol™B kit (BIOTECX Laboratories, Inc., 6023 South Loop East, Houston, Texas 77033, USA.), supplied by Bresatec. This extraction  
10 procedure is based on the method described by Chomczynski et al., Anal. Biochem. 162: 156-159 (1987), and involves homogenization in a guanidinium/phenol solution, a chloroform extraction, 2 isopropanol precipitations, and 75% EtOH washes. The RNA was stored as an EtOH precipitate  
15 at -20°C and quantitated by measuring absorption at wavelength 260 nm in water. The integrity and quantitation was confirmed by electrophoresis in agarose/formaldehyde gels. Sambrook et al. Molecular Cloning. A Laboratory Manual. Second Edition. (1989)

20 B. RT-PCR

First strand cDNA synthesis involved:

- annealing 2 $\mu$ g of total RNA from kidney, heart or liver with 120ng oligo dT primer (Gibco BRL, M-MLV Reverse Transcriptase Kit) at 65°C for 5 minutes in  
25 5 $\mu$ l of 10 mM Tris-HCl, 1mM EDTA (pH8).
- reverse transcription at 37°C for 1-2 hours in a final reaction volume of 20 $\mu$ l utilizing the M-MLV Reverse Transcriptase Kit (Gibco BRL). Each reaction contained 5mM DTT, 0.1 $\mu$ g/ $\mu$ l BSA, 1mM dNTPS, 40 U of  
30 human placental RNase Inhibitor (Bresatec), 200U of M-MLV Reverse Transcriptase and the associated RTase buffer at 1X concentration.

C. PCR Analysis of cDNA



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$\alpha$ -1,3-Gal T cDNA was detected by PCR amplification of oligo dT-primed cDNA template. Failure to generate this PCR fragment, in conjunction with the control PCR results, indicated that  $\alpha$ -1,3-Gal T mRNA was absent from the RNA preparation. To demonstrate that the  $\alpha$ -1,3-Gal T primers supported amplification of the  $\alpha$ -1,3-Gal T template, each reaction was assembled in duplicate, and one of the reactions was spiked with 0.1 fg (~100 molecules) of a 983 bp mouse  $\alpha$ -1,3-Gal T cDNA product (generated by primers 7F and mGT-3UR, spanning exon 7 to the 3' untranslated region). As a second control to demonstrate that cDNA synthesis had occurred, a ferrochelatase PCR fragment was generated from the cDNA template.

## 1. Primers:

Primers to detect  $\alpha$ -1,3-Gal T cDNA:

7F: 5'- TGGAGATCGCATTGAAGAGC 3'  
(SEQ ID NO: 20)  
(corresponding to nucleotides 889-911 within exon 7 (Figure 4))

9R2: 5'- TGGCCGCGTGGTAGTAAAAA 3'  
(SEQ ID NO: 21)  
(corresponding to nucleotides 1492-1511 within exon 9 (Figure 4))

Primers 7F and 9R2 were expected to generate a fragment of ~619 bp (Figure 23) from the cDNA template. These primers will not generate a fragment from genomic DNA possibly present in the cDNA preparation, since the primers span two large introns.

mGT-3UR: 5'- GGGTTTGGTTTGGATTGTT 3'  
(SEQ ID NO: 22)

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(corresponding to nucleotides 1866-1888 within the 3' untranslated region; Figure 4).

5 This primer was used with primer 7F to generate the DNA fragment used in the control spike PCRs.

Primers to detect mouse ferrochelatase cDNA (EcoRI linkers, underlined):

FC-F: 5'- CTGAATTCATGTTAAACATGGGAGGCCCC 3'  
(SEQ ID NO: 23)  
10 (corresponding to nucleotides 215-235, Taketani et al., J. Biol.Chem. 265: 19377-80 (1990)).

gFC-R: 5'- CTGAATTCTGCCCCACTCCCTGCCGATG 3'  
(SEQ ID NO: 24)  
15 (corresponding to nucleotides 888-908, Taketani et al., J. Biol.Chem. 265: 19377-80 (1990)).

These primers were expected to generate a 709 bp fragment (Figure 23). These primers will not generate a  
20 fragment from genomic DNA possibly present in the cDNA preparation, since the primers span five introns.

Reaction volumes were 50  $\mu$ l, consisting of 4  $\mu$ l of the first strand cDNA synthesis reaction, 100 ng of each primer, 2 mM MgCl<sub>2</sub>, 0.3 mM dNTPS, 2U of Taq-Polymerase  
25 (Bresatec) and Taq reaction buffer (Bresatec) at 1X concentration. Reactions were heated at 94°C for 2 min, then 29 cycles of 94°C for 15 sec, 58°C for 30 sec and 72°C for 1 min followed by single steps of 72°C for 4 min and 4°C for 5 min. A 10  $\mu$ l aliquot of each PCR was  
30 electrophoresed on a 2% agarose gel and DNA fragments were visualized on a UV light box after staining the gel with ethidium bromide.

Figure 24 shows the PCR fragments generated from RNA isolated from kidney (K), heart (H) and liver (L) of a

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wild-type mouse, and mice heterozygous or homozygous for the interrupted  $\alpha$ -1,3-Gal T allele. Figure 24(i) shows that the 709 bp ferrochelatase fragment was generated from each of the cDNA preparations, indicating that cDNA  
5 template was produced from the reverse transcription reaction, and was available for the  $\alpha$ -1,3-Gal T gene primers. The 619 bp  $\alpha$ -1,3-Gal T fragment was present in each of the reactions spiked with the 983 bp  $\alpha$ -1,3-Gal T cDNA product (Figure 24(ii)), indicating that amplification  
10 of the  $\alpha$ -1,3-Gal T cDNA (spike) template had occurred.

In the reactions that were not spiked (Figure 24 (iii)), the 619 bp  $\alpha$ -1,3-Gal T fragment was detected in cDNAs synthesized from the wild-type and heterozygous RNAs. This indicates that  $\alpha$ -1,3-Gal T mRNA is present in the  
15 kidney, heart and liver of the wild-type and heterozygous mice. The 619 bp fragment was not detected in the unspiked homozygous KO reactions, indicating that  $\alpha$ -1,3-Gal T mRNA is not synthesized in the homozygous KO mice.

## II. TEST FOR EXPRESSION OF THE GAL EPITOPE IN HOMOZYGOUS 20 KNOCKOUT MICE USING ANTI-GAL ANTIBODIES WITH FLUORESCENCE-ACTIVATED CELL SORTING (FACS)

### A. Solutions

Solutions 1 to 5 are 10x isotonic.

1. 1.68M NaCl (948.21g/l) Dry salts overnight in  
25 hot oven before weighing
2. 1.68M KCl (125g/l) Dry salts overnight in hot oven before weighing
3. 1.12M CaCl<sub>2</sub> (165g/l CaCl<sub>2</sub>·2H<sub>2</sub>O) Dry salts overnight in hot oven before weighing
- 30 4. 1.68M MgSO<sub>4</sub> (414g/l MgSO<sub>4</sub>·7H<sub>2</sub>O) Do not dry in hot oven
5. Potassium phosphate buffer pH 7.2:

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a) 1.68M  $\text{KH}_2\text{PO}_4$  (229 g/L)b) 1.12M  $\text{K}_2\text{HPO}_4$  (226 g/L  $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$  or 195 g/l  $\text{K}_2\text{HPO}_4$ )

Potassium phosphate buffer is prepared by  
 5 mixing together equal volumes of solutions a) and b). To  
 pH the buffer, remove a small sample, dilute 1:50 and  
 read on pH meter.

6. Hepes buffer 1M (CSL, Melbourne Australia)

7. KDS BSS:

10 Add stock solutions in the following order to  
 double-distilled water (DDW):

	Stock	Ratio of Solutions
	DDW	1210
	NaCl	121
15	KCL	3
	$\text{CaCl}_2$	3
	$\text{MgSO}_4$	1
	Potassium phosphate buffer	2
	Hepes	20

20 Filter sterlise, store at 4°C

8. KDS/BSS/2%HSA/0.02% azide:

	KDS/BSS	244.5ml
	Human serum albumin (CSL, Melbourne, Australia)	5ml
25	10% Na azide in MT-PBS	0.5ml

9. FITC dilution: Dilute 7.5ul FITC-IgG to 600ul  
 with KDS/BSS

10. Red cell lysis buffer:

0.168M  $\text{NH}_4\text{Cl}$  in double distilled water

30 11. 4% paraformaldehyde (PFA)

Solutions:

A.	$\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$	22.6 g/L
B.	NaOH	25.2 g/L
C.	40% paraformaldehyde:	

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- 1) 4 g paraformaldehyde (BDH, Kilsyth, Australia, #29447) dissolved in 10ml double distilled water. Heat 70°C 2 hours on stirrer in fume hood and a few drops of 2M NaOH are added until the solution becomes clear.
- 2) 0.54 g glucose is then added.
- 3) Store RT in light proof bottle.
- D. Add together 83 ml of A + 17 ml of B.
- E. Final 4% PFA fixative solution: 90 ml of D + 10 ml of C. pH 7.4 - 7.6; adjust pH with 1M HCl.

12. Hanks Balanced Salt Solution (Ca and Mg free) (HBBS):

15	KCL	400mg
	KH <sub>2</sub> PO <sub>4</sub>	60mg
	NaCl	8g
	NaHCO <sub>3</sub>	350mg
	Na <sub>2</sub> HPO <sub>4</sub> ·2H <sub>2</sub> O	68mg
20	Glucose	1g
	H <sub>2</sub> O	to 1 liter

adjust to pH 7.0; filter sterilize

13. Sheep antihuman IgG and IgM fluorescein isothiocyanate (FITC) F(ab)<sub>2</sub> fragments (Silenus, Hawthorn, Australia):

B. Methods

1. Eye bleed mice, collect 300-400ul into pre-chilled Ependorf tube, store on ice, add EDTA 20mg/ml to give final concentration of 2mg/ml.
2. Transfer blood (including appropriate human controls) to 10ml plain tube and add 10ml red cell lysis buffer (0.168M NH<sub>4</sub>Cl) pre-warmed to 42°C; incubate for several minutes or until cells have lysed.
3. Pellet cells by centrifugation (800 x g, 7 min, 4°C).
4. Resuspend cells in 10ml KDS/BSS/2% HSA/0.02% NaN<sub>3</sub>
5. Pellet cells as above; repeat steps 4 & 5.
6. Resuspend cells in 1000ul KDS/BSS/2% HSA/0.1% NaN<sub>3</sub>; transfer aliquots to V bottom FACS tubes.

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- 7 Pellet cells as above.
8. Resuspend cells in 100ul KDS/BSS/2% HSA/0.1%  
NaN<sub>3</sub>
9. Add 50ul of purified anti-GAL antibody (see  
5 Example 1, above), normal human serum (NHS) or HBBS/2%  
HSA/0.1% NaN<sub>3</sub> and incubate 45 min.
10. Add 2ml KDS/BSS/2% HSA/0.02% NaN<sub>3</sub>; centrifuge  
cells as above.
11. Add 50ul of a 1:80 dilution of sheep antihuman  
10 IgG or IgM FITC F(ab)<sub>2</sub> fragment (Silenus).
12. Add 2ml KDS/BSS/2% HSA/0.02% NaN<sub>3</sub>; centrifuge  
cells as above.
13. Resuspend cells in 300ul KDS/BSS/2% HSA/0.02%  
NaN<sub>3</sub>.
- 15 14. Transfer samples to plastic round-bottom FACS  
tubes and add 3 ul of propidium iodide (100ug/ml);  
samples are now ready for analysis; keep on ice.
15. Analyse on Beckman FACS scan using peripheral  
blood lymphocyte settings.

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C. Results

The results of these experiments are given below:

	median channel fluorescence (log scale)	peak channel fluorescence (log scale)
5	MOUSE 129 (Normal) PBL + FITC anti- IgG alone (neg. control)	9
	MOUSE 19 PBL (wild type) GAL IgG	286
10	MOUSE 21 PBL (Gal KO) GAL IgG	15
15	MOUSE 129 (Normal) PBL + FITC anti- IgM alone (neg. control)	1
	MOUSE 19 PBL (wild type) GAL IgM	167
20	MOUSE 21 PBL (Gal KO) GAL IgM	18
25	MOUSE 129 PBL (normal) PBL + FITC IgG alone (neg. control)	9
	MOUSE 129 PBL (normal) GAL IgG	328
30	MOUSE 9 PBL (Gal KO) GAL IgG	9

The results of human anti-Gal binding to human peripheral blood lymphocytes (negative control) are not shown but were negative. These experiments demonstrate that human anti-Gal (IgG and IgM) antibodies bind to peripheral blood cells of the homozygous  $\alpha 1,3$  galactosyltransferase knockout mice (mouse 21 and mouse 9) very weakly if at all. This confirms the expected lack of the galactose  $\alpha 1,3$  galactose (GAL) epitope in

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such mice. In contrast, peripheral blood cells of normal mice (mouse 129 and mouse 19) of the same strain display clear binding of anti-Gal antibodies.

5      III. TEST FOR EXPRESSION OF THE GAL EPIOTOPE IN HOMOZYGOUS  
KNOCKOUT MICE USING IB<sub>4</sub> LECTIN WITH FACS

IB<sub>4</sub> Lectin has an exclusive affinity for terminal  $\alpha$ -D-galactosyl residues, and is demonstrated below to be useful for characterizing the knockout mice.

### A. Solutions

- 10 1. 4% paraformaldehyde (see above)
2. Mouse Tonicity PBS (MT-PBS)
- Na<sub>2</sub>HPO<sub>4</sub> 2.27g
- NaH<sub>2</sub>PO<sub>4</sub>·2H<sub>2</sub>O 0.62g
- NaCl 8.7g
- 15 Make up to 1 liter with DDW
3. Dead Cell Removal Buffer (DCRB):
- 4.5 g Sorbitol
- 7.6 g Glucose monohydrate, (6.93 g if
- anhydrous)
- 20 -12.5 ml KDS/BSS
- Make up to 100 ml with DDW
- Filter, store at 4°C
- Open only under sterile conditions
- 25 4. KDS/BSS (Mouse Tonicity, Hepes Buffered
- Balanced Salt Solution pH 7.2) (see above)
5. Red cell lysis buffer (see above)
6. KDS/BSS/2%HSA/0.02%azide (see above)
7. Hanks Balanced Salt Solution (Ca and Mg
- free) (see above)

## 30 B. Methods

1. Remove spleen, hold with curved forceps and collect splenocytes by injecting with a 27 gauge needle bent at 90°C, injecting (2.5 ml syringe) 100-200



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- ul buffer into the spleen two or three times. Using the flat surface of the bent needle massage cells out of holes made in spleen. Repeat injections and removal of cells until no cells remain in capsule.
- 5           2.    Transfer splenocytes to 10ml tube and centrifuge to pellet cells (500xg, 7 min, 4°C).
3.    Remove supernatant and add 3ml red cell lysis buffer pre-warmed to 42°C; incubate for several minutes or until cells have lysed. Underlay with 1ml
- 10 HIFCS (heat inactivated fetal calf serum) and stand on ice 5 minutes. Top to 10ml with KDS BSS/10% HIFCS.
4.    Centrifuge as above.
5.    Resuspend cells in 3ml dead cell removal buffer; mix well with pipette.
- 15           6.    Pass through a glass pipette plugged with cotton wool and collect cells into a 10ml tube. Don't force cells through, allow to drain under gravity.
7.    Underlay cells with 1 ml BSS/10% HIFCS.
8.    Centrifuge as above.
- 20           9.    Remove supernatant.
10.   Centrifuge as above; repeat steps 4 & 5.
11.   Add 0.5 ml cold 4% paraformaldehyde (PFA).
12.   Incubate on ice for 5 min with intermittent mixing.
- 25           13.   Add 2 ml ice cold HBBS and centrifuge as above.
14.   Repeat washings with 2ml and then 1ml HBBS.
15.   Resuspend cells in 100ul KDS/BSS/2%
- 30 HSA/0.1%  $\text{NaN}_3$ ; transfer to V bottom FACS tubes.
16.   Add FITC IB4 lectin (Sigma, Cat. No. L 2895), 50ul at 20ug/ml, or 50ul HBBS; incubate on ice for 30 min.
17.   Add 2ml KDS/BSS/2% HSA/0.1%  $\text{NaN}_3$ ; spin
- 35 cells as above.

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18. Resuspend cells in 300ul KDS/BSS/2% HSA/0.1% NaN<sub>3</sub>.

19. Transfer samples to plastic round-bottom FACS tubes; samples are now ready for analysis; keep on ice.

20. Analyse on FACS scanner using peripheral blood lymphocyte setting.

C. Samples

1. Mouse 129 splenocytes alone
2. Mouse 129 splenocytes + IB<sub>4</sub> lectin
3. human PBL alone
4. Human PBL + IB<sub>4</sub> lectin

D. Results

Results of these experiments are given below:

	mean fluorescence channel (log scale)	median fluorescence channel (log scale)	peak fluorescence channel (log scale)
splenocytes alone (autofluorescence)	1	1	1
mouse 19 (wild type) splenocytes	252	58	16
mouse 21 (KO mouse) splenocytes	3	2	1

The results demonstrate that IB<sub>4</sub> lectin binds spleen cells of the homozygous  $\alpha$ 1,3 galactosyltransferase gene targeted (Gal KO) mouse (mouse 21) very weakly if at all. This confirms the expected lack of the galactose  $\alpha$ 1,3 galactose (GAL) epitope in such mice. In contrast, peripheral blood cells of a normal mouse (mouse 19) of the same strain binds IB<sub>4</sub> lectin strongly.

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IV. IMMUNOHISTOLOGICAL ASSESSMENT OF MOUSE TISSUES FOR THE PRESENCE OF THE GAL EPITOPE USING ANTI-GAL ANTIBODIES.

A. Reagents

- 5        1.    TBS: Tris Buffered Saline  
          NaCl                                8g  
          KCl                                 0.2g  
          Tris base                          3g  
          - dissolve in 800ml distilled water. Adjust pH  
10 to 8.0 with 1 M HCl. Adjust volume to 1L. Sterilise by autoclaving. Store at RT.
2.    Blocking buffer:  
              - TBS + 2% bovine serum albumin (BSA) + 10% rabbit serum:
- 15        3.    Peroxidase conjugates:  
              DAKO (Denmark) peroxidase (POD) conjugated to rabbit anti-human IgG (fragment) and DAKO (Denmark) peroxidase (POD) conjugated to rabbit anti-human IgM (fragment).
- 20        Conjugates were both separately pre-absorbed on 10% mouse liver powder at 4°C overnight, then centrifuged at 18,000xg for 10 minutes in a Biofuge and then at 30 psi for 30 min in a Beckman airfuge. Conjugated antisera were diluted 1/50 in 2% blocking buffer (TBS + 2% BSA +  
25 2% rabbit serum) with 16% normal mouse serum.
4.    Mouse liver powder preparation:  
              As modified from Antibodies, a Laboratory Manual Ed Harber and David Lane, Cold Spring Harbour Laboratories (1988) p663:
- 30        a)    Prepare a fine suspension of mouse liver in mouse tonicity phosphate buffered saline (MT-PBS). Mash liver through a sieve with a 5 ml plunger. Discard any fibrous tissue. One gram of tissue should be resuspended in approximately 1 ml MT-PBS.

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b) Transfer the tissue/saline suspension to ice for 5 min.

c) Add 8 ml of acetone ( $-20^{\circ}\text{C}$ ) (Univar 6, Ajax Chemicals) for 10 minutes. Mix vigorously.

5 Incubate on ice for 30 minutes with occasional vigorous mixing.

d) Collect the precipitate by centrifugation at 10,000g (9,000 rpm in Sorvall RC-5B refrigerated superspeed centrifuge). Spin for 10 minutes.

10 e) Resuspend the pellet with fresh acetone ( $-20^{\circ}\text{C}$ ) and mix vigorously. Allow to sit on ice for 10 minutes.

f) Centrifuge at 10,000g for 10 minutes. Transfer the pellet to a clean piece of filter paper.  
15 Spread the precipitate and allow to air-dry at room temperature.

g) After the pellet is dry, transfer it to an airtight container. Remove any large pieces that will not break into a fine powder. Dessicate and store at  
20  $4^{\circ}\text{C}$ .

Yield as approximately 10-20% of the original wet weight. To use acetone powders, add to a final concentration of 1%. Incubate for 30 min at  $4^{\circ}\text{C}$ .

Spin at 10,000g for 10 minutes. (13,000 rpm in Biofuge)

25 5. DAB/ $\text{H}_2\text{O}_2$ /Imidazole:

Peroxidase substrate: 3,3'-Diaminobenzidine tetrahydrochloride (DAB) (Sigma, Missouri)

- 1 tablet DAB (take out of fridge 10 min before use)

30 - 1 tablet urea  $\text{H}_2\text{O}_2$  (Sigma, Missouri)

- add to 15 ml tris HCL, pH 7.6 + 0.01M imidazole (0.0102g), (Sigma, Missouri)

- make up immediately before use

6. Tris HCL:

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1.211g Tris in 200ml double distilled water pH

7.6

## 7. Animal serum sources:

Mouse and rabbit sera were obtained in-house  
(St. Vincent's Hospital, Dept, of Clinical Immunology).  
Sheep serum was obtained from the University of  
Melbourne Veterinary Clinic and Hospital, Werribee,  
Australia.

## 8. Harris Haematoxylin:

Haematoxylin C.I. 75290 (BDII, Poole, U.K.  
#34037) 10g

Absolute ethanol 200ml  
Potassium alum 200g  
double distilled water 2000ml

glacial acetic acid 80ml

Preparation: 1. Dissolve  
haematoxylin in absolute ethanol

2. Heat to dissolve alum in double distilled  
water

3. Mix solution 1 and 2

4. Immediately before use add 80 ml 1% sodium  
iodate and 80 ml glacial acetic acid

## 9. Scott's Tap Water:

Sodium hydrogen carbonate 14 g  
MgSO<sub>4</sub> 80 g  
Tap water 4 litres

B. Methods

1. Cut 4 um sections of the relevant tissue on  
cryostat
2. Tissue should be free of cracks
3. Air dry slides for 30 min

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4. Apply 10% blocking buffer at room temp in humidified chamber, 60 min
5. Remove blocking antibody with tissue made to fine point
- 5 6. Apply 1st antibody, anti-GAL, or 2% blocking buffer as control, 50ul, ensure no air bubbles and incubate at room temp in humidified chamber for 30 min
- 10 7. Wash off with Tris buffered saline (TBS) 3 times 2 minutes washes
8. Apply second antibody 1:50 peroxidase (POD) conjugated rabbit anti-human IgG and IgM (DAKO, Denmark); incubate 30 min at room temp in humidified chamber
- 15 9. Wash off with Tris buffered saline (TBS) 3 times 3 minute washes
10. Wash off with TBS as above
11. Incubate DAB/H<sub>2</sub>O<sub>2</sub>/imidazole for 10 minutes
12. Wash in water
- 20 13. Stain with haemotoxylin C - 10 seconds
14. Wash in water
15. Place in Scotts tap water for 15 seconds
16. Wash in water
17. Wash in absolute alcohol (x3) (Univar 214, Ajax chemicals)
- 25 18. Wash in absolute xylene (x3) (Univar 577, Ajax chemicals)
19. Coverslip using automatic coverslip machine (Tissue Tek)
- 30 Controls:
  1. Buffer only + POD conjugated rabbit anti-human IgM (negative)

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2. Buffer only + POD conjugated rabbit anti-human IgG (negative)
3. Human kidney (negative)
4. Pig renal cortex (positive)

## 5 Samples:

1. Mouse 129 SV (control) kidney
2. mouse 9 (Gal Knockout) kidney
3. mouse 21 (Gal Knockout) kidney

C. Results

## 10 KIDNEY

	GLOMERULI	ENDOTHELIUM	comments
MOUSE 129 anti-IgM	POSITIVE	POSITIVE	
MOUSE 9 anti-IgM	NEGATIVE	NEGATIVE	weak adventitial staining
15 MOUSE 21 anti-IgM	NEGATIVE	NEGATIVE	weak adventitial staining
MOUSE 129 anti-IgG	POSITIVE	POSITIVE	
20 MOUSE 9 anti-IgG	NEGATIVE	NEGATIVE	
MOUSE 21 anti-IgG	NEGATIVE	NEGATIVE	
POD conjugated antibody alone	ALL NEGATIVE	ALL NEGATIVE	

25 These results indicate that human anti-Gal IgG and IgM antibodies do not bind kidney tissue of the  $\alpha 1,3$  galactosyltransferase gene targeted (Gal KO) mice (mouse 21 and mouse 9). This confirms that lack of the galactose  $\alpha 1,3$  galactose (GAL) epitope in the gene

30 targeted (KO) mice. In contrast, these antibodies react strongly with the endothelium of blood vessels and the glomeruli of a normal mouse of the same strain (129).

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V. IMMUNOHISTOLOGICAL EXAMINATION OF MOUSE TISSUES  
USING IB<sub>4</sub> LECTIN

A. Reagents

1. Blocking buffer: TBS + 2% BSA + 10% sheep serum
- 5 2. FITC IB<sub>4</sub> (Sigma, Missouri, USA #L-2895)  
1 mg diluted in 1 ml HBBS to give stock  
solution, then dilute to final volume of 20 ug/ml in TBS  
+ 2% BSA + 2% sheep serum
3. Peroxidase anti-FITC
- 10 Boehringer anti-fluorescein POD Fab fragments;  
dilute 1/300 in 2% blocking buffer
4. DAB/H<sub>2</sub>O<sub>2</sub>/Imidazole - see above
5. Tris HCL - see above
6. Animal serum sources - see above
- 15 7. Harris Haematoxylin - see above
8. Scott's Tap Water - see above

B. Methods

1. Preparation of Sections; same as Section 4B,  
steps 1-7 above.
- 20 2. Apply 50 µl FITC conjugated IB<sub>4</sub>  
(Sigma # 1-2894) 20 µg/ml, incubate in a  
humidified chamber for 30 minutes.
3. Wash with TBS, 3 minutes (x3).
- 25 4. Apply 50 µl per oxidase conjugated anti - FITC  
Fab fragments (Boehringer Mannheim), diluted 1-3-- with  
TBS + 2% BSA + 2% sheep serum. Incubate for 30 minutes in  
humidified chamber.
5. Wash with TBS, 3 minutes (x3).
6. Processing for microscopy - same as Section IVB  
30 steps 14-22.

Controls



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1. Buffer only + POD anti-FITC (negative)
2. Human kidney (negative)
3. Pig renal cortex (positive)

**Samples 1st Experiment**

- 5 1. Mouse 129 SV normal mouse heart liver kidney lung
2. mouse 6 wild type heart liver kidney lung
3. mouse 7 heterozygote KO heart liver kidney lung
4. mouse 9 homozygous KO heart liver kidney lung

**Samples 2nd Experiment**

- 10 1. mouse 19 wild type heart liver kidney lung
2. mouse 20 heterozygote KO heart liver kidney lung
3. mouse 21 homozygous KO heart liver kidney lung

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C. Results**Kidney**

5

10

	GLOMERULI	ENDOTHELIUM
HUMAN	NEGATIVE	NEGATIVE
PIG	POSITIVE	POSITIVE
129 MOUSE	POSITIVE	POSITIVE
MOUSE 6	POSITIVE	POSITIVE
MOUSE 7	POSITIVE	POSITIVE
MOUSE 9	NEGATIVE	NEGATIVE
MOUSE 19	POSITIVE	POSITIVE
MOUSE 20	POSITIVE	POSITIVE
MOUSE 21	NEGATIVE	NEGATIVE
anti-FITC alone	ALL NEGATIVE	ALL NEGATIVE

**Liver**

15

20

	ENDOTHELIUM	BILE DUCT
129 MOUSE	POSITIVE	POSITIVE
MOUSE 6	POSITIVE	POSITIVE
MOUSE 7	POSITIVE	POSITIVE
MOUSE 9	NEGATIVE	NEGATIVE
MOUSE 19	POSITIVE	POSITIVE
MOUSE 20	POSITIVE	POSITIVE
MOUSE 21	NEGATIVE	NEGATIVE
anti-FITC alone	ALL NEGATIVE	ALL NEGATIVE

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**Heart**

5

	ENDOTHELIUM	PERINUCLEAR	ENDO-MYOCARDIUM
129 MOUSE	POSITIVE	POSITIVE	POSITIVE
MOUSE 6	POSITIVE	POSITIVE	POSITIVE
MOUSE 7	POSITIVE	POSITIVE	POSITIVE
MOUSE 9	NEGATIVE	NEGATIVE	NEGATIVE
MOUSE 19	POSITIVE	POSITIVE	POSITIVE
MOUSE 20	POSITIVE	POSITIVE	POSITIVE
MOUSE 21	NEGATIVE	NEGATIVE	NEGATIVE
anti-FITC alone	ALL NEGATIVE	ALL NEGATIVE	ALL NEGATIVE

10 **Lung**

15

	ENDOTHELIUM	BRONCHI	PARENCHYMA
129 MOUSE	POSITIVE	POSITIVE	POSITIVE
MOUSE 6	POSITIVE	POSITIVE	POSITIVE
MOUSE 7	POSITIVE	POSITIVE	POSITIVE
MOUSE 9	NEGATIVE	NEGATIVE	NEGATIVE
MOUSE 19	POSITIVE	POSITIVE	POSITIVE
MOUSE 20	POSITIVE	POSITIVE	POSITIVE
MOUSE 21	NEGATIVE	NEGATIVE	NEGATIVE
anti-FITC alone	ALL NEGATIVE	ALL NEGATIVE	ALL NEGATIVE

20 These results indicate that IB<sub>4</sub> lectin does not bind kidney, heart, liver or lung tissue of the  $\alpha$ 1,3 galactosyltransferase gene targeted (Gal KO) homozygous mice (mouse 21 and mouse 9). This confirms the lack of the galactose  $\alpha$ 1,3 galactose (GAL) epitope in the gene targeted (KO) mice. In contrast these antibodies react

25 strongly with the tissues of a normal mouse and heterozygous KO mice (mouse 129, mouse 6, mouse 7, mouse 19, mouse 20) of the same strain.

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# VI. RESISTANCE OF SPLEEN CELLS FROM KNOCKOUT MICE TO LYSIS BY HUMAN SERUM

Lysis of spleen cells by human serum was tested through use of a <sup>51</sup> chromium release assay. See in general

5 Example 4, above.

A. Preparation of Mouse Splenocytes - Shortman, K.J. et al, Immunological Methods. 1:273-287 (1972).:

-Dissect out spleen, avoid damaging outer membranes and carefully remove mesentery tissue and fat.

10 -Place in petri dish, with 1 ml RPMI 1640 (Gibco BRL) /10% Heat-inactivated foetal calf serum (HI-FCS). (Heat-inactivation = 40 Min at 56°C).

-Gently tease out cells into petri dish, collect and centrifuge 500xg, 5 min, 4°C

15 -Remove RPMI/10% HIFCS, gently resuspend cells in 3 ml 0.9% NH<sub>4</sub>Cl (0.168M), using a Pasteur pipette. (Use Pasteur pipettes or wide-bore pipettes for all re suspension and transfer procedures)

20 -Transfer to 10 ml tube, underlay with 1 ml HIFCS, stand on ice, 5 min.

-Transfer supernatant to clean tube, centrifuge 500xg, 7 min, 4°C

-Discard supernatant, re-suspend cells in 3 ml dead cell removal buffer, mix well with pipette.

25 -Pass through cotton wool plug in glass pipette (under gravity, do not force through), collect cells into 10 ml tube.

-Underlay cells with 1 ml HI-FCS.

-Centrifuge 500xg, 7 min, 4°C

30 -Remove supernatant, re-suspend cells in 50 µl RPMI, 10% HI-FCS. Store cells on ice.

## B. Preparation of Serum:

Human - Collect whole blood from a pool of normal donors; allow to stand at room temp. for 2  
35 hours.

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Wring the clot with an 'Orange stick'; spin  
Remove and pool serum. Store half at  $-70^{\circ}\text{C}$  in  
3 ml aliquot's (normal human serum); heat-  
inactivate the other half, see below.

- 5 Fetal calf serum - purchased from Gibco BRL, and stored  
at  $-20^{\circ}\text{C}$ .

C. Cell Counting:

1. Add 5  $\mu\text{l}$  cells to 95.0  $\mu\text{l}$  RPMI, 10% HI-FCS
2. Remove 10  $\mu\text{l}$  cells, add 10  $\mu\text{l}$  Acridine  
10 Orange/Et Br solution, (Lee, S.K. et al. Eur J. Immunol.  
1975. 5: 259-262)
3. Count cells, (viable = green, deads = orange).
4. Cell viability should be approx. 90-100 %
5. Calculate cell number.

- 15 D.  $^{51}\text{Chromium}$  Labelling:

Cell Type	Incubation conditions	
	Time	Amount $^{51}\text{Cr}/10^7$ cells
Freshly prepared cells: (eg., splenocytes or 20 lymphocytes)	~2 hours	~150-300 $\mu\text{Ci}$
Cultured Cells:	~1 hour	~100 $\mu\text{Ci}$

Labelling:

- Combine:
- 25 - cells ( $2 \times 10^7$ )
  - ( $^{51}\text{Cr}$ ) Sodium Chromate in 0.9% NaCl solution  
(the volume added depends on cell type as indicated above  
and on the specific activity of the - ( $^{51}\text{Cr}$ ) Sodium  
Chromate).
  - 30 - RPMI/2% HIFCS up to a total of 200  $\mu\text{l}$

Incubate at  $37^{\circ}\text{C}$  for time shown above with gentle  
agitation every 15 min.

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## E. Washing

- Place 4ml HI-FCS into 10ml tube and carefully layer labelling reaction on top with a swirling motion; centrifuge 5 min, 500xg, 4°C.
- 5 -Remove top two layers with a careful circular motion using a glass pipette.
- Resuspend cells in 1ml RPMI/2% HI FCS
- Pellet cell suspension through another 4 ml HI FCS
- 10 -Resuspend cell pellet in 1ml RPMI/2% HI FCS, store on ice.

## F. Release Assay:

- Perform assay in 96-well microtitre plate (ICN-FLOW).
- Assay should be set up in quadruplicate.
- 15 -Assay is performed in a total volume of 180 µl.

## Assay:

	NHS	*HI-NHS	16% SDS	CELLS	RPMI/2% HIFCS
MAX Release	-	90µl	22.5µl	25µl	42.5µl
Spont. Release	-	90		25	65µl
20 5% NHS	9µl	81		25	65
10% NHS	18	72		25	65
20% NHS	36	54		25	65
30% NHS	54	36		25	65
40% NHS	72	18		25	65
25 50% NHS	90	-		25	65

\*HI = heat inactivated

-All volumes indicated are in µl

-Reaction components are added to the plate in the order: RPMI, Serum and <sup>51</sup>Cr-labelled cells.

- 30 -Cover plate with plate-sealer
- Incubate, 4 hours, 37°C.
- Spin plate, 1500 rpm, 5 min.
- Remove plate-scaler, remove 80 µl from each wall, count released chromium on gamma counter.
- 35 -Calculate specific lysis for each well according to the formula:

$$\% \text{ Specific Lysis} = \frac{(\text{Test cpm} - \text{Spontaneous release cpm}) \times 100}{(\text{Maximal release cpm} - \text{Spontaneous release cpm})}$$

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Calculate mean and standard deviation for each experimental point. Graph % Human serum (X axis) against % Specific lysis (Y axis) for each type of cell (wild type, heterozygote KO and homozygous KO)

5                   The results of these experiments are depicted in Figure 25. The results indicate that spleen cells from a homozygous knockout mouse are relatively resistant to lysis by human serum, in comparison to spleen cells derived from mice heterozygous for the interrupted allele or from wild-type mice.

#### EXAMPLE 16

##### Generation of Knockout Animals Through Microinjection of Eggs

Transgenic animals are generated routinely by microinjection of DNA into the pronuclei of fertilised eggs. Generally this technology results in the random integration of the transgene in the genome. However, conventional transgenic technology has resulted in homologous recombination between the injected transgene and the endogenous gene. See, for example, Brinster et al., Proc. Nat. Acad. Sci. USA 86: 1087-91 (1989). Described below are procedures for inactivating the  $\alpha$ -1,3-Gal T gene in pigs through microinjection of eggs with gene targeting constructs.

#### 25 I. GENE TARGETING CONSTRUCTS

The frequency of homologous recombination in embryos is improved if the gene targeting constructs are prepared with isogenic DNA. Therefore the "knock out" constructs are prepared from DNA isolated from the boar used to fertilize the oocytes used for microinjection. DNA is isolated from the tail or ear tissue, and genomic fragments from both  $\alpha$ -1,3-Gal T alleles of the boar, encompassing exons 8 & 9 are cloned using long range PCR or conventional genomic library technologies. Clones for each of the  $\alpha$ -1,3-Gal T alleles

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are identified using restriction fragment length polymorphism identification and DNA sequencing. Constructs to target both alleles are made by interrupting the coding sequence of exon 9, either by deletion or by inserting a heterologous DNA fragment. The constructs contain at least 8 kb of homologous DNA to promote efficient homologous recombination.

Various approaches can be used to detect gene targeting events, depending on the strategies used in designing the knockout constructs. Several such approaches, and the corresponding strategies for construction of constructs, are provided below:

a) PCR of Genomic DNA:

Homologous DNA on one side of the interrupting DNA fragment is constructed to be less than 1 kb, allowing PCR amplification of a short diagnostic fragment. (Amplification of small fragments generally is relatively efficient).

b) Reverse Transcription/PCR:

A deletion of about 100 bp within exon 9 is made, allowing synthesis of a shortened  $\alpha$ -1,3-Gal T mRNA in correctly targeted cells. The shortened mRNA is detected by RT/PCR, using primers that amplify a fragment extending from exon 8 and encompassing the deletion site.

c) Green Fluorescent Protein (GFP) gene expression:

GFP is a protein from the bioluminescent jelly fish *Aequorea victoria*. It absorbs blue light (395 nm) and fluoresces to emit green light (509 nm). GFP is a useful marker for gene expression. Chafie et al., Green Fluorescent Protein as a Marker for Gene Expression. Science 263: 802-5 (1994). The  $\alpha$ -1,3-Gal T gene is interrupted within exon 9 by in-frame insertion of the GFP coding region. Expression of the GFP gene (with



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resulting fluorescence at 509 nm) is driven by the  $\alpha$ -1,3-Gal T gene promoter in correctly targeted cells.

## II. GENERATING EMBRYOS FOR MICROINJECTION

Fertilized embryos are generated as described by Nottle et al., (1993). Proc Aust Soc for Reproductive Biol 26, 33. The protocol involves:

- a) Sperm from the boar providing DNA for the targeting construct is collected and stored frozen in liquid N<sub>2</sub>.
- 10 b) Superovulation of donor gilts:

Gilts are mated at the second oestrus, and aborted between days 25-40 days of gestation to synchronise the subsequent oestrus cycles. Abortion is achieved by intramuscular injection of 1 mg cloprostenol

15 (a prostaglandin F2 $\alpha$  analogue), followed by a second 0.5 mg injection 24 hours later. Gilts are superovulated by injection of 1000 i.u. equine chorionic gonadotrophin (eCG) or pregnant mare serum gonadotrophin at the time of the second cloprostenol injection, and a subsequent

20 injection 72 hours later of 500 i.u. human chorionic gonadotrophin (hCG).

- c) Fertilization:

Superovulated gilts are artificially inseminated 20-30 hours after the hCG injection, followed by a second

25 insemination 2-4 hours later, with semen from the boar that provided DNA for the targeting construct.

- d) Embryo collection:

Embryos are collected surgically 50-56 hours after hCG injection prior to fusion of the pronuclei. Oviducts are

30 flushed with 15-20 ml phosphate saline buffer containing 1% fetal calf serum. One-cell embryos are recovered by searching oviductal flushings using low magnification microscopy.

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### III. MICROINJECTION OF EMBRYOS

Embryos are centrifuged at 12000 x g for 8 min to stratify the cytoplasm and allow the pronuclei to be visualised, and held in Dulbecco's Minimal Essential Medium with 25 mM Hepes and 5 mg/ml bovine serum albumin. Pronuclei are injected, using differential interference contrast optics, with 4-10 picolitres of DNA (10 ng/ $\mu$ l) in PBS. Gene targeting with isogenic DNA is maximized by coinjecting both allelic constructs derived from the boar into the male pronucleus.

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IV. TRANSFER OF INJECTED EMBRYOS TO RECIPIENT  
GILTS

The oestrus cycles of recipient gilts are  
synchronized with those of donors. The recipients are  
5 mated and aborted using the protocol described above, and  
injected with 500 i.u. eCG. Injected embryos are  
transferred surgically (20-40 per oviduct) to recipients  
on the same day that they are collected from donor gilts.

V. SCREENING FOR HOMOLOGOUS RECOMBINATION

10 Homologous recombinants can be detected by  
analysis of tissue from the born piglets. Screening  
procedures involve PCR technology, the precise strategy  
depending on the design of the gene targeting construct.  
Because many  $\alpha$ -1,3-Gal T mRNA molecules are synthesized  
15 from a single  $\alpha$ -1,3-Gal T gene in expressing cells, the  
RT/PCR approach can be more sensitive than PCR  
amplification of genomic DNA. The RT/PCR screening  
strategy relies on successful transcription of the  
interrupted gene and relative stability of the shortened  
20 mRNA.

Alternatively, constructs that promote  
expression of heterologous genes (eg: GFP) in correctly  
targeted cells allow embryos to be screened at the  
blastocyst stage for marker gene expression (i.e.: GFP  
25 expression can be detected by measuring fluorescence  
within blastocysts at 509 nm). The microinjected embryos  
are cultured *in vitro* until blastocyst development,  
screened for fluorescence, and fluorescing embryos  
transferred into recipients.

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## EXAMPLE 17

A Novel Variant of Leukemia Inhibitory Factor (LIF)

Previous reports have demonstrated the existence of two forms of murine LIF. The original form (from the D transcript) was expressed and commercialized by AMRAD Corporation Ltd (Kew Victoria, Australia). The protein product derived from this transcript (hereinafter "D-LIF") is sold commercially by AMRAD as "ESGRO". Another form of LIF (hereinafter "M-LIF"), derived from an alternative transcript, is described in US Patent Application No. 07/994,099 and in Rathjen et al., Cell 62: 1105-14 (1990). The present inventors have now found a third transcript of LIF (hereinafter "T-LIF") which is found in ES cells and in human teratocarcinoma-derived cell lines such as the GCT 27 teratocarcinoma-derived cell lines described by Pera et al., Differentiation 42: 10 (1989).

The T-LIF protein is found intracellularly in contrast to the other two forms of LIF which are both extracellular. The transcript was cloned using the RACE PCR technique (see below) from murine ES cells and human GCT 27 teratocarcinoma-derived cell lines, and sequenced using standard methods. The presence of the T-LIF transcript was confirmed by PCR analysis of ES cell mRNA and RNA' ase protection on GCT 27 RNA. The transcript comprises a novel first exon, located in the first intron of the LIF gene, spliced to the known exon 2 and exon 3 sequences. The mouse nucleotide sequence (SEQ ID NO: 25) and deduced amino acid sequence (SEQ ID NO: 26) are set out in Figure 26. The human nucleotide sequence (SEQ ID NO: 31) and deduced amino acid sequence (SEQ ID NO: 32) are set out in Figure 27.

When expressed in a COS cell expression system, the murine T-LIF transcript produces a 17 kD

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protein that is unglycosylated (D-LIF is glycosylated in the Golgi during the secretion process) (Figure 28). Translation of T-LIF initiates at the first in-frame initiation codon (ATG) in exon 2 to produce a protein of 158 amino acids. The protein is 45 amino acids shorter than the unprocessed D-LIF protein and 22 amino acids shorter than the mature D-LIF product generated by cleavage of the signal sequence. Because the T-LIF protein does not contain a signal sequence, it does not leave the cell and is unglycosylated. The T form of LIF is efficacious in preventing the differentiation of ES cells in culture.

#### METHODS

##### RACE cDNA CLONING

Cytoplasmic RNA (10 $\mu$ g) from CP1 murine ES cells (Bradley et al., Nature 309: 255-56 (1984)) was reverse transcribed from the oligonucleotide 5'ACACGGTACTTGTTGCA-3' (SEQ ID NO: 27), which hybridizes to residues 500-484 of the murine LIF cDNA. The RNA was added to 20 pmol of primer and 2 $\mu$ l of 10x annealing buffer (500mM Tris-HCl (pH 8.0), 60mM MgCl<sub>2</sub>, 400mM KCl) in a total volume of 16 $\mu$ l, heated to 85°C for 5 min, and cooled slowly to room temperature. The elongation reaction was carried out as described by Frohman et al. (Proc. Natl. Acad. Sci. USA 85: 8998-9002 (1988)). Excess oligonucleotide was removed by gel filtration through a 2ml Sephacryl S-400 (Pharmacia) column equilibrated with 0.05 x TE (TE = 10mM Tris-HCl pH 7.6, 1.0mM EDTA). Fractions of 50 $\mu$ l corresponding to the cDNA radioactive peak were pooled, concentrated by vacuum centrifugation, and resuspended in 23 $\mu$ l of H<sub>2</sub>O. To tail the 3'-end of the cDNA with dG residues, 3 $\mu$ l of 10mM dGTP and 6 $\mu$ l of 5 x tailing buffer (Bethesda Research Laboratories) were added and the mixture was incubated at

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37°C for 60 min. and then at 70°C for 15 min. After ethanol precipitation, the cDNA template was resuspended in 500µl H<sub>2</sub>O.

PCR was carried out using a mouse LIF specific oligonucleotide, 5'-TTCTGGTCCCGGGTGATATTGGTCA-3' (residues 389-365) (SEQ ID NO: 28), and an anchor oligonucleotide, 5'-CCATGGCCTCGAGGGCCCCCCCCCCCCCCC-3' (SEQ ID NO: 29). PCR was carried out in a final volume of 50µl containing 7µl of the cDNA template and 34pmol of each oligonucleotide. Reaction conditions were as recommended by Perkin-Elmer Cetus, with a final concentration of 1.5mM MgCl<sub>2</sub>. DNA was denatured prior to the addition of Taq polymerase (Perkin-Elmer Cetus) by heating the reaction mixture to 94°C for 5 min. Each PCR cycle (35 in total) consisted of denaturation for 2 min at 94°C, annealing for 2 min at 55°C, and elongation for 3 min at 72°C. After the final elongation (30 min at 72°C), samples were ethanol precipitated, digested with SmaI and XhoI and analyzed by agarose gel electrophoresis. DNA was purified from agarose gels using Geneclean and cloned into SalI- and SmaI- digested TST7 19U (Stratagene). Suitable recombinant plasmids were purified by the rapid boiling method.

Double-stranded sequencing was performed with Sequenase version 2.0 (USB) according to the manufacturers recommendations.

#### BIOLOGICAL ASSAY FOR LIF ACTIVITY

An undifferentiated, murine ES cell culture (MBL5; Pease et al., Dev. Biol. 141: 344-52 (1990), between passages 15 and 30) is trypsinized and made into a single cell suspension. The cells are pelleted by centrifugation and resuspended in complete ES Cell Medium without LIF (DMEM (without Hepes), 10% FCS, 1mM βME, 1mM glutamine). The cells are then seeded into

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24-well microtitre plates at  $5 \times 10^2$  cells/16 mm well containing 1 ml of ES Cell Medium without LIF.

The complete T-LIF open reading frame was reconstructed from the PCR product and inserted into the COS cell expression vector pXMT2 as described by Rathjen et al., Cell 62: 1105-14 (1990). The plasmid used for transfection of COS cells is shown in Figure 29. The COS cells were transfected by electroporation. Supernatants from COS cells expressing T-LIF were added to the above ES cells in various dilutions (1/5, 1/10, 1/50, 1/100, 1/50, 1/1000) and incubated for 4 days in an incubator with 10% CO<sub>2</sub>. Controls used supernatants from COS cells expressing D-LIF (pDR1, Rathjen et al., Cell 62: 1105-14 (1990)).

LIF activity is assessed as present if cells morphologically resemble ES-cells after 4 days and are distinct from the controls incubated without any form of LIF. The ES-cells are also stained for alkaline phosphatase as undifferentiated ES-cells are positive for this marker.

Even though T-LIF is produced intracellularly, sufficient numbers of cells lyse to give significant amounts of LIF activity in the culture supernatants. If the COS cells expressing T-LIF are lysed, more LIF activity is released.

#### PCR DETECTION OF T-LIF TRANSCRIPT

PCR was carried out on ES cell cDNA (prepared as described above except that the cDNA was not tailed with dG). PCR conditions were as described above except that 2mM MgCl<sub>2</sub> was used in the reactions. The oligonucleotides 5'-CACCTTTCGCTTTCCT-3' (SEQ. ID NO. 30) and 5'-TTCTGGTCCCGGGTGATATTGGTCA-3' (SEQ. ID. NO 28) were used at 80 picograms/reaction. Products of the PCR reaction were ethanol precipitated as described above,

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separated electrophoretically on a 2% agarose gel and transferred to a nylon membrane for detection using Southern hybridization (Figure 30). The probe was the full length D-LIF transcript isolated from pDR1 (Rathjen  
5 et al., Cell 62: 1105-14 (1990). The control experiment is designed to detect all LIF transcripts using internal primers 5'-TTCTGGTCCCGGGTGATATTGGTCA-3' (SEQ. ID. NO 28) and 5'-CTGTTGGTTCTGCACTGGA-3' (SEQ. ID. NO. 33).

The foregoing detailed description has  
10 been provided for a better understanding of the invention only and no unnecessary limitation should be understood therefrom as some modifications will be apparent to those skilled in the art without deviating from the spirit and scope of the appended claims.

15 What is claimed is:



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1. A purified and isolated nucleic acid molecule comprising a nucleic acid sequence selected from the group consisting of (1) the porcine nucleic acid sequence depicted in Figure 4 (SEQ ID NO: 7), (2) a  
5 sequence corresponding to the sequence of (1) within the scope of the degeneracy of the genetic code, (3) a sequence that encodes a porcine polypeptide having  $\alpha$ -1,3 galactosyltransferase activity and that hybridizes under standard high stringency conditions with a sequence  
10 complementary to the sequence of (1) or (2), and (4) a sequence complementary to the sequence of (1), (2) or (3).
2. A host cell that is transformed with the nucleic acid molecule of claim 1.
- 15 3. A porcine  $\alpha$ -1,3 galactosyltransferase encoded by the nucleic acid molecule of claim 2.
4. A DNA construct useful for inactivating the porcine  $\alpha$ -1,3 galactosyltransferase gene by insertion of a desired DNA sequence into an insertion site of said  
20 gene, comprising said desired DNA sequence flanked by first and second homology sequences, said first and second homology sequences being, respectively, sufficiently homologous to first and second genomic sequences flanking said insertion site to allow for  
25 homologous recombination of said DNA construct with said porcine  $\alpha$ -1,3 galactosyltransferase gene when said DNA construct is introduced into a porcine cell having said  $\alpha$ -1,3 galactosyltransferase gene.

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5. The DNA construct of claim 4, wherein said insertion site is within exon 4, exon 7, exon 8 or exon 9 of the porcine  $\alpha$ -1,3 galactosyltransferase gene.
6. The DNA construct of claim 4, wherein said  
5 desired DNA sequence is selected from the group consisting of the neo<sup>R</sup> gene, the hyg<sup>R</sup> gene and the thymidine kinase gene.
7. The DNA construct of claim 6, wherein said  
10 desired DNA sequence is bordered at the 5' and 3' ends by FRT DNA elements, and wherein stop codons for each of the three reading frames have been inserted 3' to the desired DNA sequence.
8. A DNA construct useful for inactivating  
15 the murine  $\alpha$ -1,3 galactosyltransferase gene by insertion of a desired DNA sequence into an insertion site of said gene, comprising said desired DNA sequence flanked by first and second homology sequences, said first and second homology sequences being, respectively,  
20 sufficiently homologous to first and second genomic sequences flanking said insertion site to allow for homologous recombination of said DNA construct with said murine  $\alpha$ -1,3 galactosyltransferase gene when said DNA construct is introduced into a murine cell having said  $\alpha$ -1,3 galactosyltransferase gene.
- 25 9. The DNA construct of claim 8, wherein said insertion site is within exon 4, exon 7, exon 8 or exon 9 of the murine  $\alpha$ -1,3 galactosyltransferase gene.
10. The DNA construct of claim 8, wherein said desired DNA sequence is selected from the group

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consisting of the neo<sup>R</sup> gene, the hyg<sup>R</sup> gene and the thymidine kinase gene.

11. The DNA construct of claim 10, wherein said desired DNA sequence is bordered at the 5' and 3' ends by FRT DNA elements, and wherein stop codons for each of the three reading frames have been inserted 3' to the desired DNA sequence.

12. A method for generating a mammalian totipotent cell having at least one inactivated  $\alpha$ -1,3 galactosyltransferase allele, said totipotent cell derived from a mammalian species having a functional  $\alpha$ -1,3 galactosyltransferase gene, comprising:

(a) providing a plurality of cells characterized as totipotent cells of said mammalian species;

(b) introducing into said totipotent cells a nucleic acid construct effective for inactivating said  $\alpha$ -1,3 galactosyltransferase gene by insertion of a desired DNA sequence into an insertion site of said gene through homologous recombination; and

(c) identifying a totipotent cell having at least one inactivated  $\alpha$ -1,3 galactosyltransferase allele.

13. The method of claim 12 in which said totipotent cell is a murine ES cell.

14. The method of claim 12 in which said totipotent cell is a murine egg.

15. The method of claim 12 in which said totipotent cell is a porcine ES cell.

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16. The method of claim 12 in which said totipotent cell is a porcine PGC.

17. The method of claim 12 in which said totipotent cell is a porcine egg.

5 18. A method for generating a mammal lacking a functional  $\alpha$ -1,3 galactosyltransferase gene, said mammal belonging to a species having a functional  $\alpha$ -1,3 galactosyltransferase gene, comprising:

10 (a) providing a mammalian totipotent cell having at least one inactivated  $\alpha$ -1,3 galactosyltransferase allele, said totipotent cell derived from a mammalian species having a functional  $\alpha$ -1,3 galactosyltransferase gene;

15 (b) manipulating said totipotent cell such that mitotic descendants of said cell constitute all or part of a developing embryo;

(c) recovering a neonate derived from said embryo; and

20 (d) raising and breeding said neonate to obtain a mammal homozygous for said inactivated  $\alpha$ -1,3 galactosyltransferase allele.

19. The method of claim 18, wherein said totipotent cell is a murine ES cell and said manipulating comprises injecting said ES cell into the blastocyst cavity of a murine blastocyst and implanting said injected blastocyst into a murine recipient female.

20. The method of claim 18, wherein said totipotent cell is a murine egg, and said manipulating comprises implanting said egg into a murine recipient female.

30

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21. The method of claim 18, wherein said totipotent cell is a porcine ES cell and said manipulating comprises injecting said ES cell into the blastocyst cavity of a porcine blastocyst and implanting  
5 said injected blastocyst into a porcine recipient female.

22. The method of claim 18, wherein said totipotent cell is a porcine ES cell and said manipulating comprises injecting said ES cell into a porcine morula.

10 23. The method of claim 18, wherein said totipotent cell is a porcine ES cell and said manipulating comprises co-culture of said ES cell with a zona pellucida-disrupted porcine morula.

15 24. The method of claim 18, wherein said totipotent cell is a porcine ES cell and said manipulating comprises fusing said ES cell with an enucleated porcine zygote.

20 25. The method of claim 18, wherein said totipotent cell is a porcine egg, and said manipulating comprises implanting said egg into a porcine recipient female.

25 26. A mammal lacking a functional  $\alpha$ -1,3 galactosyltransferase gene, said mammal belonging to a species having a functional  $\alpha$ -1,3 galactosyltransferase gene, said mammal produced by the method of claim 18.

27. The mammal of claim 26, wherein said mammal is a mouse.

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28. The mammal of claim 26, wherein said mammal is a pig.

29. A non-naturally occurring mammal lacking a functional  $\alpha$ -1,3 galactosyltransferase gene, said mammal  
5 belonging to a species having a functional  $\alpha$ -1,3 galactosyltransferase gene.

30. The mammal of claim 29, wherein said mammal is a mouse.

31. The mammal of claim 29, wherein said  
10 mammal is a pig.

32. A purified and isolated nucleic acid molecule comprising a nucleic acid sequence selected from the group consisting of (1) the nucleic acid sequence depicted in Figure 26 (SEQ ID NO: 25), (2) a sequence  
15 corresponding to the sequence of (1) within the scope of the degeneracy of the genetic code, (3) a sequence that encodes murine T-LIF and that hybridizes under standard high stringency conditions with a sequence complementary to the sequence of (1) or (2), and (4) a sequence  
20 complementary to the sequence of (1), (2) or (3).

33. A host cell that is transformed with the nucleic acid molecule of claim 32.

34. A murine T-LIF polypeptide encoded by the nucleic acid molecule of claim 32.

25 35. A purified and isolated nucleic acid molecule comprising a nucleic acid sequence selected from the group consisting of (1) the nucleic acid sequence depicted in Figure 27 (SEQ ID NO: 31), (2) a sequence

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corresponding to the sequence of (1) within the scope of the degeneracy of the genetic code, (3) a sequence that encodes human T-LIF and that hybridizes under standard high stringency conditions with a sequence complementary to the sequence of (1) or (2), and (4) a sequence complementary to the sequence of (1), (2) or (3).

36. A host cell that is transformed with the nucleic acid molecule of claim 35.

37. A human T-LIF polypeptide encoded by the nucleic acid molecule of claim 35.

38. A method for eliminating or reducing hyperacute rejection of non-primate mammalian cells, tissues and organs by human serum, comprising adding, to said human serum, a physiologically acceptable amount of galactose or a saccharide in which the terminal carbohydrate is an  $\alpha$  galactose linked at position 1, prior to exposure of said human serum to said non-primate cells, wherein said amount of galactose or saccharide is sufficient to reduce or eliminate said hyperacute rejection.

39. The method of claim 38, wherein said saccharide is selected from the group consisting of melibiose, galactose  $\alpha$ 1-3 galactose and stachyose.

40. A method for eliminating or reducing hyperacute rejection of non-primate mammalian cells, tissues and organs by human serum, comprising substantially depleting said serum of immunoglobulin.

41. A method for eliminating or reducing hyperacute rejection of non-primate mammalian cells,

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tissues and organs by human serum, comprising substantially depleting said serum of IgM antibodies.

42. A method for eliminating or reducing hyperacute rejection of non-primate mammalian cells by human serum, comprising substantially depleting said serum of anti-GAL IgM and IgG antibodies.

43. A method for eliminating or reducing hyperacute rejection of non-primate mammalian cells by human serum, comprising substantially depleting said serum of anti-GAL IgM antibodies.

44. Affinity-treated human serum substantially free of anti-GAL antibodies.

45. Affinity-treated human serum substantially free of anti-GAL IgM antibodies.



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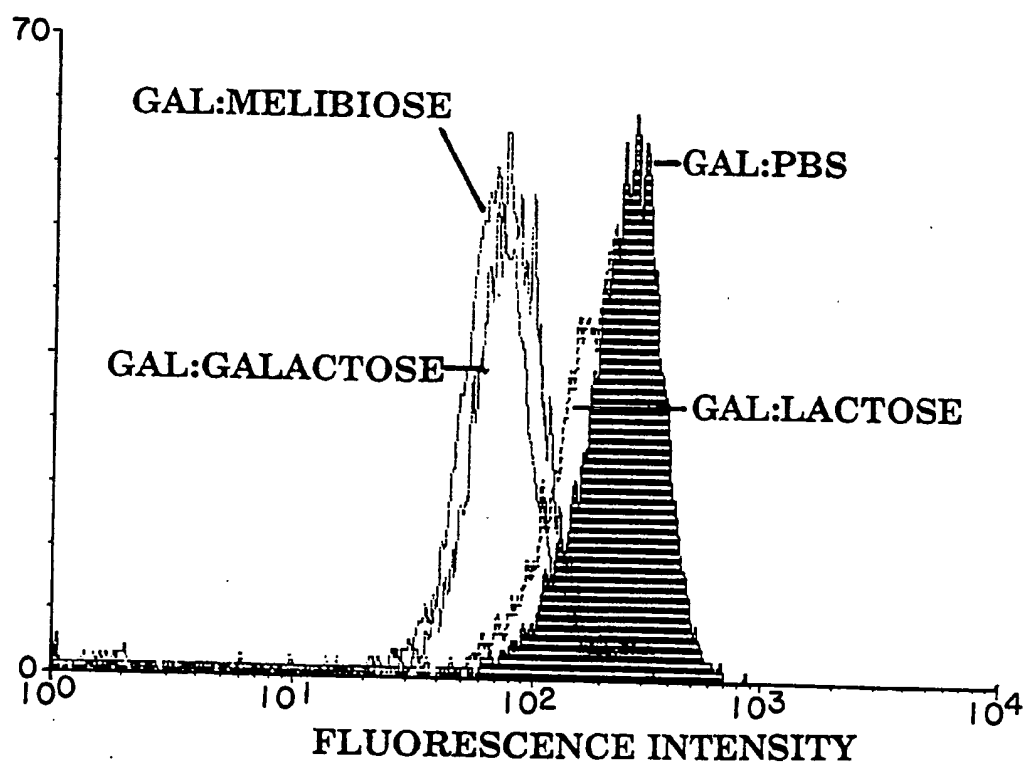


FIG. 1

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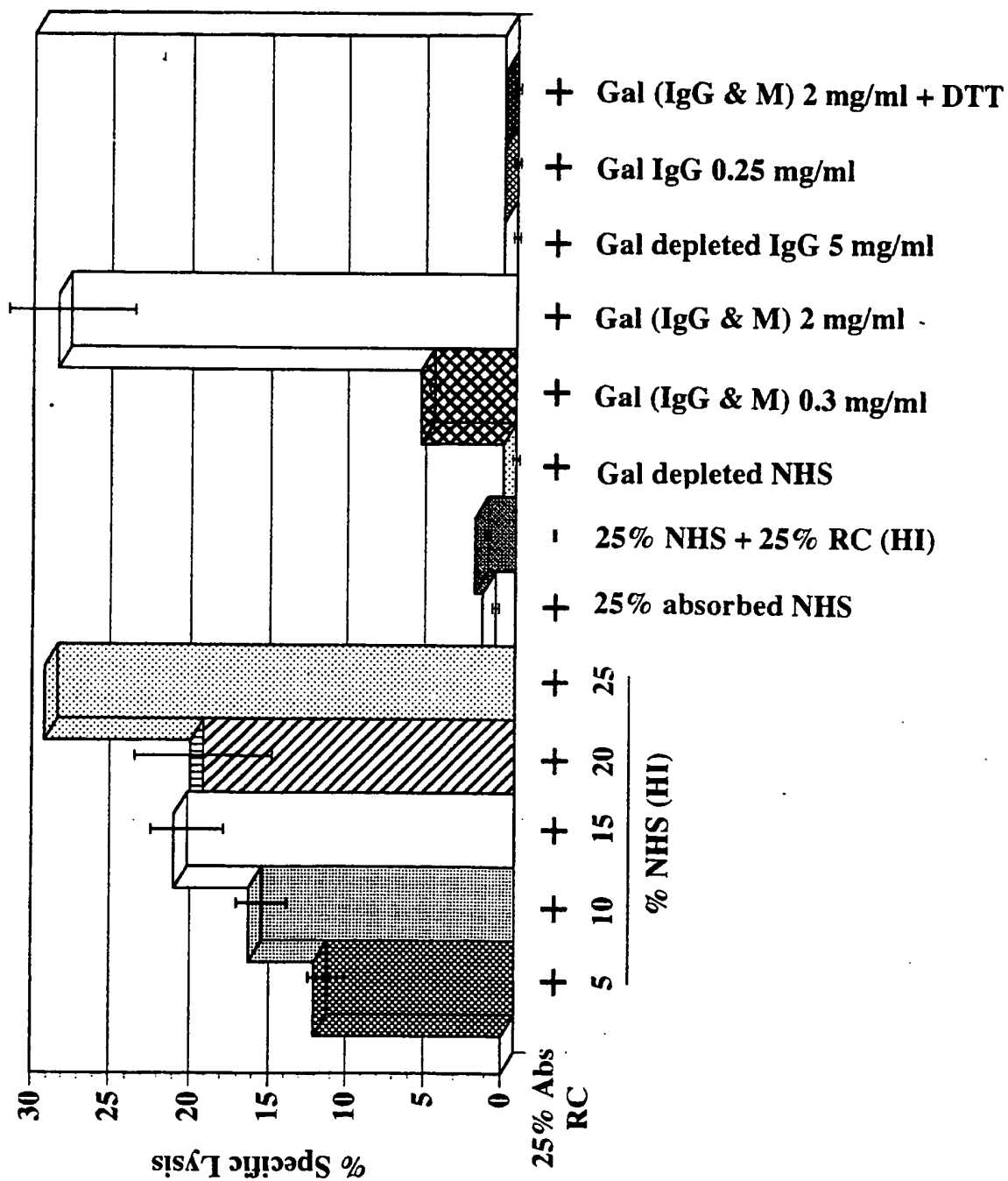


FIG. 2

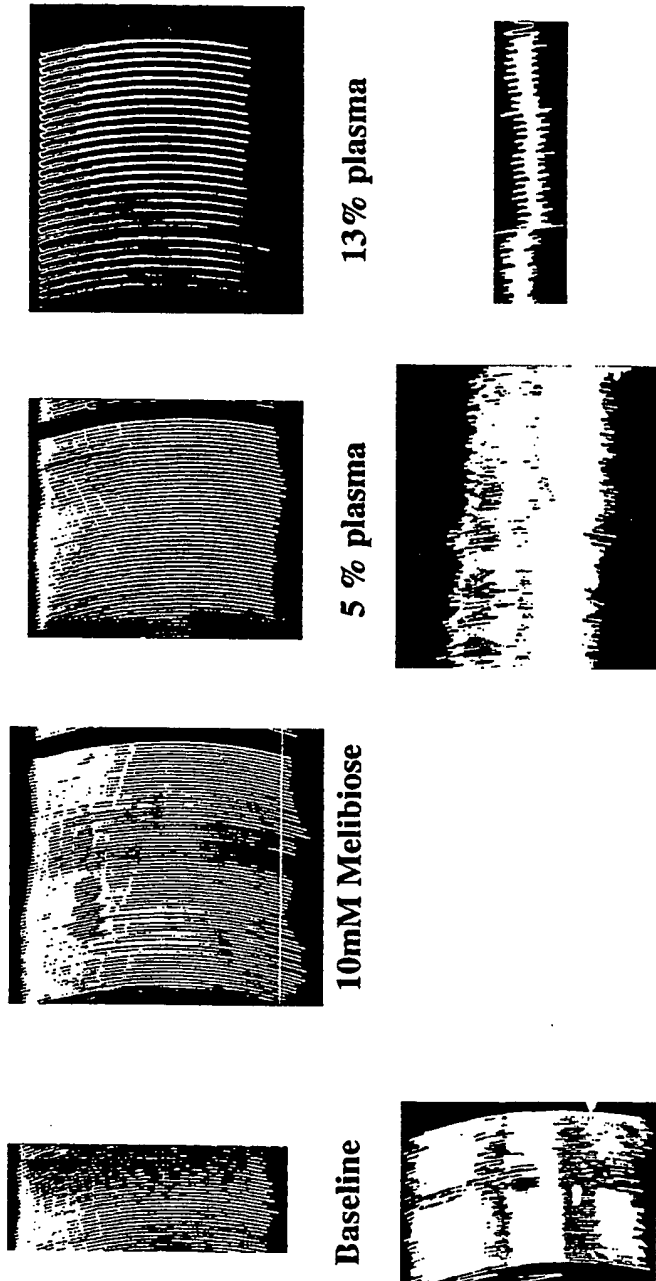


FIG. 3

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144	145	146	147	148	149	150	151	152	153	154	155	156	157	158	159	160	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180	181	182	183	184	185	186	187	188	189	190	191	192	193	194	195	196	197	198	199	200	201	202	203	204	205	206	207	208	209	210	211	212	213	214	215	216	217	218	219	220	221	222	223	224	225	226	227	228	229	230	231	232	233	234	235	236	237	238	239	240	241	242	243	244	245	246	247	248	249	250	251	252	253	254	255	256	257	258	259	260	261	262	263	264	265	266	267	268	269	270	271	272	273	274	275	276	277	278	279	280	281	282	283	284	285	286	287	288	289	290	291	292	293	294	295	296	297	298	299	300	301	302	303	304	305	306	307	308	309	310	311	312	313	314	315	316	317	318	319	320	321	322	323	324	325	326	327	328	329	330	331	332	333	334	335	336	337	338	339	340	341	342	343	344	345	346	347	348	349	350	351	352	353	354	355	356	357	358	359	360	361	362	363	364	365	366	367	368	369	370	371	372	373	374	375	376	377	378	379	380	381	382	383	384	385	386	387	388	389	390	391	392	393	394	395	396	397	398	399	400	401	402	403	404	405	406	407	408	409	410	411	412	413	414	415	416	417	418	419	420	421	422	423	424	425	426	427	428	429	430	431	432	433	434	435	436	437	438	439	440	441	442	443	444	445	446	447	448	449	450	451	452	453	454	455	456	457	458	459	460	461	462	463	464	465	466	467	468	469	470	471	472	473	474	475	476	477	478	479	480	481	482	483	484	485	486	487	488	489	490	491	492	493	494	495	496	497	498	499	500	501	502	503	504	505	506	507	508	509	510	511	512	513	514	515	516	517	518	519	520	521	522	523	52
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**FIG. 4a**

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PGTCD	351	-----	-----	-----AGC	CCTGCCCTCCT	TCTGCAGAGC	400
BOVGSTA	351	AAGGCTGCAC	CTTCGCTTCC	TCCC-----AGC	CCTGCCCTCCT	TCTGCAGAAC	400
MUSGLYTNS	351	AACCCCTGTAC	CTTCCTTTTCC	TCTGCTGAGC	CCTGCCCTCCT	TCGGCAGGCC	400
PGTCD	401	AGAGCTCACT	AGAACTT-GT	TTC-----GCC	TTTTACTCTG	GGGGGAGAGA	450
BOVGSTA	401	GGAGCTCAGT	AGAACTT-GG	TACTTTTGCC	TTTTACTCTA	GGAGGAGAGA	450
MUSGLYTNS	401	AGAGCTCGAC	AGAAGCTCGG	TTGCTTTTGCT	GTTTGCTTTG	GAGGGAACAC	450
<div style="text-align: center;"> <div style="display: inline-block; text-align: right;">Exon 2</div> <div style="display: inline-block; text-align: left;">Exon 3</div> </div>							
PGTCD	451	AGCAGAGGAT	GAG-----	-----	-----	-----	500
BOVGSTA	451	AGCAGACGAT	GAG-----	-----	-----	-----	500
MUSGLYTNS	451	AGCTGACGAT	GAGGCTGACT	TTGAACTCAA	GAGATCTGCT	TACCCCAAGTC	500
PGTCD	501	-----	-----	-----	-----	-----	550
BOVGSTA	501	-----	-----	-----	-----	-----	550
MUSGLYTNS	501	TCCTGGAAAT	AAAGGCCTGT	ACTACCTTGC	CTGGACCTAA	GATTTTCAATG	550
PGTCD	551	-----	-----	-----	-----	-----	600
BOVGSTA	551	-----	-----	-----	-----	-----	600
MUSGLYTNS	551	ATCACTATGC	TTCAAGATCT	CCATGTCAAC	AAGATCTCCA	TGTCAAGATC	600
<div style="text-align: center;"> <div style="display: inline-block; text-align: right;">Exon 3</div> <div style="display: inline-block; text-align: left;">Exon 4</div> </div>							
PGTCD	601	-----	-----	-----	-----	-----GAGAAAA	650
BOVGSTA	601	-----	-----	-----	-----	-----GAGAAAA	650
MUSGLYTNS	601	CAAGTCAGAA	ACAAGTCTTC	CATCCTCAAG	ATCTGGATCA	CAGGAGAAAA	650
PGTCD	651	TAAATGAATGT	CAAAGGAAGA	GTGGTTCTGT	CAATGCTGCT	TGTCTCAACT	700
BOVGSTA	651	TAAATGAATGT	CAAAGGAAGA	GTGATTTCTGT	CAATGCTGGT	TGTCTCAACT	700
MUSGLYTNS	651	TAAATGAATGT	CAAGGGAAGA	GTAATCCTGT	TGATGCTGAT	TGTCTCAACC	700

Start

FIG. 4b

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PGTCD BOVGSTA MUSGLYTNS	701	GTAATGGTTG	TGTTTTGGGA	ATACATCAAC	AGCCCCAGAAG	GTTCCTTTGTT	750
	701	GTCATTGTTG	TGTTTTGGGA	ATATATCCAC	AGCCCCAGAAG	GCTCTTTGTT	750
	701	GTGGTTGTCTG	TGTTTTGGGA	ATATGTCAAC	AGCCCCAGACG	GCTCTTTCTT	750
<div> <div>Exon 4</div> <div>Exon 5</div> </div>							
PGTCD BOVGSTA MUSGLYTNS	751	CTGGATATAC	CAGTCAAAAA	ACCCAGAAAGT	TGGCAGCAGT	GCTCAGAGGG	800
	751	CTGGATAAAC	CCATCAAGAA	ACCCAGAAAGT	TGGTGGCAGC	AGCATTCAGA	800
	751	GTGGATATAT	CACACAAAAA	TTCCAGAGGT	TGGTGAGAAC	AGATGGCAGA	800
<div> <div>Exon 5</div> <div>Exon 6</div> </div>							
PGTCD BOVGSTA MUSGLYTNS	801	GCTGGTGG--	-TTTCCGAGC	TGGTTTAAAC	ATGGGACTCA	CAGTTACCAC	850
	801	AGGCTGGTG	GCITCCGAGA	TGGTTTAAAC	ATG-----	--GTTACCAT	850
	801	AGGACTGGTG	GTTCCCAAGC	TGGTTTAAAA	ATGGGACCCA	CAGTTATCAA	850
<div> <div>Exon 6</div> <div>Exon 7</div> </div>							
PGTCD BOVGSTA MUSGLYTNS	851	GAAGAAGAAG	ACGCTATAGG	CAACGAAAAAG	GAACAAAGAA	AAGAAGACAA	900
	851	GAAGAAGATG	GAGACATAAA	CGAAGAAAAAG	GAACAAAGAA	ACGAAGACGA	900
	851	GAAGACAACG	TAGAAGGACG	GAGAGAAAAAG	GGTAGAAATG	GAGATCGCAT	900
<div> <div>Exon 7</div> <div>Exon 8</div> </div>							
PGTCD BOVGSTA MUSGLYTNS	901	CAGAGGAGAG	CTTCCGCTAG	TGGACTGGTT	TAATCCTGAG	AAACGCCCCAG	950
	901	---AAGCAAG	CTTAAGCTAT	CGGACTGGTT	CAACCCATTT	AAACGCCCCG	950
	901	---TGAAGAG	CCTCAGCTAT	GGGACTGGTT	CAATCCAAAG	AACCGCCCCG	950
PGTCD BOVGSTA MUSGLYTNS	951	AGGTCGTGAC	CATAACCAGA	TGGAAGGCTC	CAGTGGTATG	GGAAGGCAC	1000
	951	AGGTTGTGAC	CATGACGAAG	TGGAAGGCTC	CAGTGGTGTG	GGAAGGCAC	1000
	951	ATGTTTTGAC	AGTGACCCCG	TGGAAGGCGC	CGATTGTGTG	GGAAGGCAC	1000
PGTCD BOVGSTA MUSGLYTNS	1001	TACAACACAG	CCGTCCTTGA	TAATTATAAT	GCCAAACAGA	AAATTACCGT	1050
	1001	TACAACACAG	CCGTCCTTGA	CAATTATTAT	GCCAAACAGA	AAATTACCGT	1050
	1001	TATGACACAG	CTCTGCTGGA	AAAGTACTAC	GCCACACAGA	AACTCACTGT	1050

FIG. 4c

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		Exon 8		Exon 9	
PGTCD	1051	GGGCTTGACG	GTTTTTGCTG	TCGGAAGATA	CATTGAGCAT
	1051	CGGCTTGACG	GTTTTTGCTG	TCGGAAGATA	CATTGAGCAT
	1051	GGGCTTGACA	GTTTGTGCTG	TGGGAAAGTA	CATTGAGCAT
BOVGSTA	1101	AGTTCTTTAAT	ATCTGCAAAT	ACATACTTCA	TGGTTGGCCA
	1101	AGTTCTTTAAT	GTCTGCTAAT	AAGCACTTCA	TGGTTGGCCA
	1101	ACTTTCTGGA	GTCTGCTGAC	ATGTACTTCA	TGGTTGGCCA
MUSGLYTNS	1151	TTTTACATCA	TGGTGGATGA	TATCTCCAGG	ATGCCCTTTGA
	1151	TTTTATATCA	TGGTAGATGA	TGTCTCCAGG	ATGCCCTTTGA
	1151	TTTACGTCA	TGATAGATGA	CACCTCCCGG	ATGCCCTGTCG
PGTCD	1201	TCCCTCTGCGT	TCCTTTAAAG	TGTTTGAGAT	CAAGTCCGAG
	1201	TCCCTCTGCGC	TCCTTCAAAG	TGTTTAAGAT	CAAGCCTTGAG
	1201	CCCCTCTACAT	TCCTTACAAG	TCTTTGAGAT	CAGGTCTGAG
BOVGSTA	1251	AAGACATCAG	CATGATGCCG	ATGAAGACCA	TCGGGGAGCA
	1251	AGGACATCAG	CATGATGCCG	ATGAAGACTA	TCGGGGAGCA
	1251	AGGATATCAG	CATGATGCCG	ATGAAGACCA	TTGGGGAGCA
MUSGLYTNS	1301	CACATCCAGC	ACGAGGTGGA	CTTCCCTCTTC	TGCATGGACG
	1301	CACATCCAGC	ATGAGGTGGA	CTTCCCTTTTC	TGCATGGATG
	1301	CACATCCAGC	ACGAGGTGGA	CTTCCCTCTTC	TGCATGGACG
PGTCD	1351	CTTCCAAAAC	AACTTTGGGG	TGGAGACCCCT	GGGCCAGTCG
	1351	CTTCCAAAGAC	AAGTTTGGGG	TGGAGACCCCT	GGGCCAGTCG
	1351	CTTTCAAGAC	AACTTTCGGGG	TGGAACCTCT	GGGCCAGCTG
BOVGSTA	1400				GTGGCTCAGC
	1400				GTGGCCCCAGC
	1400				GTAGCACAGC

FIG. 4d

PGTCD	1401	TACAGGCCCTG	GTGGTACAAG	GCACATCCTG	ACGAGTTCAC	CTACGAGAGG	1450
BOVGSTA	1401	TACAAAGCCTG	GTGGTACAAG	GCAGATCCCA	ATGACTTCAC	CTACGAGAGG	1450
MUSGLYTNS	1401	TCCAGGCCCTG	GTGGTACAAG	GCCAGTCCCG	AGAAGTTCAC	CTATGAGAGG	1450
PGTCD	1451	CGGAAGGAGT	CCGCAGCCCTA	CATTCCGTTT	GGCCAGGGGG	ATTTTATTTA	1500
BOVGSTA	1451	CGGAAGGAGT	CTGCAGCATA	CATTCCCTTC	GGCGAAGGGG	ATTTTATTTA	1500
MUSGLYTNS	1451	CGGGAAGTGT	CGGCCGCGTA	CATTCCATTC	GGAGAGGGGG	ATTTTACTTA	1500
PGTCD	1501	CCACGCAGCC	ATTTTGTGGG	GAACACCCAC	TCAGGTTCTA	AACATCACTC	1550
BOVGSTA	1501	CCATGCAGCC	ATTTTGTGGG	GAACACCCAC	TCAGGTCCCTT	AACATCACTC	1550
MUSGLYTNS	1501	CCACGGGGCC	ATTTTGTGGG	GAACGCCTAC	TCACATTCCTC	AACCTCACCA	1550
PGTCD	1551	AGGAGTGCTT	CAAGGGAATC	CTCCAGGACA	AGGAAAATGA	CATAGAAGCC	1600
BOVGSTA	1551	AGGAATGCTT	CAAAGGAATC	CTCAAGGACA	AGAAAATGA	CATAGAAGCC	1600
MUSGLYTNS	1551	GGGAGTGCTT	TAAGGGGATC	CTCCAGGACA	AGAAAATGA	CATAGAAGCC	1600
PGTCD	1601	GAGTGGCATG	ATGAAAGCCA	TCTAAACAAG	TATTTAATTC	TCAACAAACC	1650
BOVGSTA	1601	CAATGGCATG	ATGAAAGCCA	TCTAAACAAG	TATTTCCCTC	TCAACAAACC	1650
MUSGLYTNS	1601	CAGTGGCATG	ATGAGAGCCA	CCTCAACAAA	TACTTCCCTT	TCAACAAACC	1650
PGTCD	1651	CACTAAAATC	TTATCCCCAG	AATACTGCTG	GGATTATCAT	ATAGGCATGT	1700
BOVGSTA	1651	TACTAAAATC	TTATCCCCCG	AATACTGCTG	GGATTATCAC	ATAGGCCTAC	1700
MUSGLYTNS	1651	CACTAAAATC	CTATCTCCAG	AGTATTGCTG	GGACTATCAG	ATAGGCCTGC	1700
PGTCD	1701	CTGTGGATAT	TAGGATTGTC	AAGATAGCTT	GGCAGAAAAA	AGAGTATAAT	1750
BOVGSTA	1701	CTGCGGATAT	TAAGCTTGTC	AAGATGCTTT	GGCAGACAAA	AGAGTATAAT	1750
MUSGLYTNS	1701	CTTCAGATAT	TAAAAGTGTC	AAGTAGCTTT	GGCAGACAAA	AGAGTATAAT	1750

FIG. 4e



PGTCD	1751	TTGGTTAGAA	ATAACATCTG	ACTTTAAATT	GTGCCAGCAG	TTTTCTGAAT	1800
BOVGSTA	1751	GTGGTTAGAA	ATAATGCTTG	ACTT-----T	GTGCCAGTAC	ATTTCTGAAT	1800
MUSGLYTNS	1751	TTGGTTAGAA	ATAATGCTTG	ACTTCAAAAT	GTG-----	--ATGGAAC	1800
				Stop			
PGTCD	1801	TTGAAAGAGT	ATTACTCTGG	CTACTTCCTC	AGAGAAGTAG	---CACTTAA	1850
BOVGSTA	1801	TTGAGAGAGT	ATTATTCCTG	CTACTTCCTC	AGAAAAGTAA	---CACTTAA	1850
MUSGLYTNS	1801	TTGACAC--T	ATTACTCTGG	CTAATTCCTC	AAACAAGTAG	CAACACTTGA	1850
PGTCD	1851	TTTTAACTTT	TAAAAAATA	CTAACAAA-	----TACCAC	CACAGTAA-G	1900
BOVGSTA	1851	TTTTAACTTA	AAAAAATA	CTAACAAA-	----GACCAA	CACAGCAA-A	1900
MUSGLYTNS	1851	TTTCAACTTT	TAAAGAA-A	CAATCAAAAC	CAAAACCCAC	TACCATGGCA	1900
PGTCD	1901	TACATATTAT	TCTTCCTTGC	AACCTTGAGC	CTTGTCAAAT	GGGAGAAATGA	1950
BOVGSTA	1901	TACATATTAT	TTCTCCTTGT	AACCTTGAGC	CTTGTAATAC	GGGAGAAATGA	1950
MUSGLYTNS	1901	AACAGATGAT	TTCTCCT-GA	CACCTTGAGC	CT-GTAATAT	GTGAGAAAGA	1950
PGTCD	1951	CTCTGTGG--	--TAATCAGA	TGTAAATTCC	CAGTGATTTT	.....	2000
BOVGSTA	1951	ACCTGTGG--	--TAATCAGA	TGTAAATTCC	CAGTGATTTT	TTACCTATTT	2000
MUSGLYTNS	1951	GTCATATGCA	AGTAATCAGG	TATAAATTCT	CAATGATTTT	TTATATATTC	2000
PGTCD	2001	.....	.....	.....	.....	.....	2050
BOVGSTA	2001	TTGGTTGTGG	GGCGGGGAA	TGGATACACC	ATCAGTTGAA	CC.....	2050
MUSGLYTNS	2001	TGGGTCITGG	GAAAACTTGA	TTCTAGAAAT	CAAAATTAAT	TTGACAAAGG	2050
PGTCD	2051	.....	.....	.....	.....	.....	2100
BOVGSTA	2051	.....	.....	.....	.....	.....	2100
MUSGLYTNS	2051	AAAAGCAGAT	GCCGGAAACT	TCTTCCCAGT	CTGTCATACA	ATTCACCACT	2100

FIG. 4f

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[illegible]

**FIG. 4g**

PGTCD	2451	.....	.....	.....	.....	.....	.....	.....	.....	.....
BOVGSTA	2451	.....	.....	.....	.....	.....	.....	.....	.....	.....
MUSGLYTNS	2451	GTTACAAACA	CAGACAGCTT	TCTTCAGGAT	GAATAACAGC	AGGAATTGAA	.....	.....	.....	.....
PGTCD	2501	.....	.....	.....	.....	.....	.....	.....	.....	.....
BOVGSTA	2501	.....	.....	.....	.....	.....	.....	.....	.....	.....
MUSGLYTNS	2501	TGGAAAGTGT	GTTTCATTTTG	TTTTGCCCAA	ATTGTATTCA	TGCTGTTTAGC	.....	.....	.....	.....
PGTCD	2551	.....	.....	.....	.....	.....	.....	.....	.....	.....
BOVGSTA	2551	.....	.....	.....	.....	.....	.....	.....	.....	.....
MUSGLYTNS	2551	TTTGTGTGTT	GAGCCCTGTG	GAGAGGGTGT	GACTGTATCA	GGGAAGGAGA	.....	.....	.....	.....
PGTCD	2601	.....	.....	.....	.....	.....	.....	.....	.....	.....
BOVGSTA	2601	.....	.....	.....	.....	.....	.....	.....	.....	.....
MUSGLYTNS	2601	GTACCCTCAGC	GGACTGAGGA	CCAGCACCCCT	ATTATATCAG	AAGACAATCT	.....	.....	.....	.....
PGTCD	2651	.....	.....	.....	.....	.....	.....	.....	.....	.....
BOVGSTA	2651	.....	.....	.....	.....	.....	.....	.....	.....	.....
MUSGLYTNS	2651	CTCATCATCA	GGTCCCTACCT	ACAACCTGCT	CTGAACCTCC	GAGTTCCTCA	.....	.....	.....	.....
PGTCD	2701	.....	.....	.....	.....	.....	.....	.....	.....	.....
BOVGSTA	2701	.....	.....	.....	.....	.....	.....	.....	.....	.....
MUSGLYTNS	2701	GCCCATCGTG	TTCCAGTGTG	GGGGCCTGTA	TGGAGCAGGT	GAATAAAGAC	.....	.....	.....	.....
PGTCD	2751	.....	.....	.....	.....	.....	.....	.....	.....	.....
BOVGSTA	2751	.....	.....	.....	.....	.....	.....	.....	.....	.....
MUSGLYTNS	2751	AAAGCCCCCT	GTCACATGAC	CTCATTTCCC	CTGCTCTAGT	ACTATGCAAG	.....	.....	.....	.....

**FIG. 4h**

PGTCD	2801	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
BOVGSTA	2801	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .
MUSGLYTNS	2801	TGTGACAGCC	AGCCAGCCAG	ATGTACTGGA	CAACATAGGA	ACCGACTTTA	ACCGACTTTA	ACCGACTTTA	2850
PGTCD	2851	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	2900
BOVGSTA	2851	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	2900
MUSGLYTNS	2851	TGGCAATGGG	AGCCGCAGTC	ACTACAACGG	AGCTGCTGAA	GTTCTGTTC	GTTCTGTTC	GTTCTGTTC	2900
PGTCD	2901	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	2950
BOVGSTA	2901	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	2950
MUSGLYTNS	2901	CCC GC TCTGA	GAGCCTGCAG	GAGCCCCCTGT	ATAGGTGGTT	CTCAACCTAT	CTCAACCTAT	CTCAACCTAT	2950
PGTCD	2951	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	3000
BOVGSTA	2951	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	3000
MUSGLYTNS	2951	GGGTCGCGAC	CCCTTTGGGA	AGTGTTAAAT	GACCC TTTC A	CAGGTGTCCC	CAGGTGTCCC	CAGGTGTCCC	3000
PGTCD	3001	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	3050
BOVGSTA	3001	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	3050
MUSGLYTNS	3001	CTAAGACGGT	TAAAAAACAT	AGATA TTTC C	ACTCTGACTG	GTAACAGTAG	GTAACAGTAG	GTAACAGTAG	3050
PGTCD	3051	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	3100
BOVGSTA	3051	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	. . . . .	3100
MUSGLYTNS	3051	CAGAATTACA	GTTATGAAAT	AGCAAGGGAA	ATAATTCTGG	GTTCTGTTC	GTTCTGTTC	GTTCTGTTC	3100

**FIG. 4i**

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				Ex4 ▼ Ex5	Ex5 ▼ Ex6	
PGT[Frame 1]	1	MNVKGRVLS	MLLVSTVMVV	FWEYINSPEG	SLFWIYQSKN	PEVG-SSAQR 50
BGT[Frame 1]	1	MNVKGKVLS	MLVVSTVIVV	FWEYIHSPEG	SLFWINPSRN	PEVGGSSIQK 50
MGT[Frame 1]	1	MNVKGKVILL	MLIVSTVVVV	FWEYVNSPDG	SFLWIYHTKI	PEVGENRWQK 50

		Ex6 ▼ Ex7		Ex7 ▼ Ex8		
PGT[Frame 1]	51	GWFFPSWFNN	GTHSYHEED	AIGNEKEQK	EDNRGELPLV	DWFNPEKRPE 100
BGT[Frame 1]	51	GWWLPRWFNN	G---YHEEDG	DINEEKEQRN	ED-ESKLKLS	DWFNPFKRPE 100
MGT[Frame 1]	51	DWWFPSWFKN	GTHSYQEDNV	EGRREK-GRN	GDRIEEPQLW	DWFNPKNRPD 100

				Ex8 ▼ Ex9		
PGT[Frame 1]	101	VVTITRWKAP	VWEGTYNRA	VLDNYNAKQK	ITVGLTVFAV	GRYIEHYLEE 150
BGT[Frame 1]	101	VVTMTKWKAP	VWEGTYNRA	VLDNYYAKQK	ITVGLTVFAV	GRYIEHYLEE 150
MGT[Frame 1]	101	VLTVTPWKAP	IVWEGTYDTA	LLEKYYATQK	LTVGLTVFAV	GKYIEHYLED 150

PGT[Frame 1]	151	FLISANTYFM	VGHKVIFYIM	VDDISRMPLI	ELGPLRSFKV	FEIKSEKRWQ 200
BGT[Frame 1]	151	FLTSANKHFM	VGHPVIFYIM	VDDVSRMPLI	ELGPLRSFKV	FKIKPEKRWQ 200
MGT[Frame 1]	151	FLESADMYFM	VGHRVIFYVM	IDDTSRMPVV	HLNPLHSLQV	FEIRSEKRWQ 200

PGT[Frame 1]	201	DISMMRMKTI	GEHILAHIQH	EVDFLFCMDV	DQVFQNNFGV	ETLGQSVACL 250
BGT[Frame 1]	201	DISMMRMKTI	GEHIVAHIQH	EVDFLFCMDV	DQVFQDKFGV	ETLGESVACL 250
MGT[Frame 1]	201	DISMMRMKTI	GEHILAHIQH	EVDFLFCMDV	DQVFQDNFGV	ETLGQLVACL 250

PGT[Frame 1]	251	QAWWYKAHPD	EFTYERRKES	AAYIPFGQGD	FYYHAAIFGG	TPTQVLNITQ 300
BGT[Frame 1]	251	QAWWYKADPN	DFTYERRKES	AAYIPFGEGD	FYYHAAIFGG	TPTQVLNITQ 300
MGT[Frame 1]	251	QAWWYKASPE	KPTYERRELS	AAYIPFGEGD	FYYHAAIFGG	TPTHILNLTR 300

PGT[Frame 1]	301	ECFKGILQDK	ENDIEAEWHD	ESHLNKYLIL	NKPTKILSPE	YCWDYHIGMS 350
BGT[Frame 1]	301	ECFKGILKDK	KNDIEAQWHD	ESHLNKYFLL	NKPTKILSPE	YCWDYHIGLP 350
MGT[Frame 1]	301	ECFKGILQDK	KHDIEAQWHD	ESHLNKYFLF	NKPTKILSPE	YCWDYQIGLP 350

PGT[Frame 1]	351	VDIKIVKIAW	QKKEYNLVRN	NI*.....	.....	400
BGT[Frame 1]	351	ADIKLVKMSW	QTKEYNVVRN	NV*.....	.....	400
MGT[Frame 1]	351	SDIKSVKVAW	QTKEYNLVRN	NV*.....	.....	400

FIG. 5

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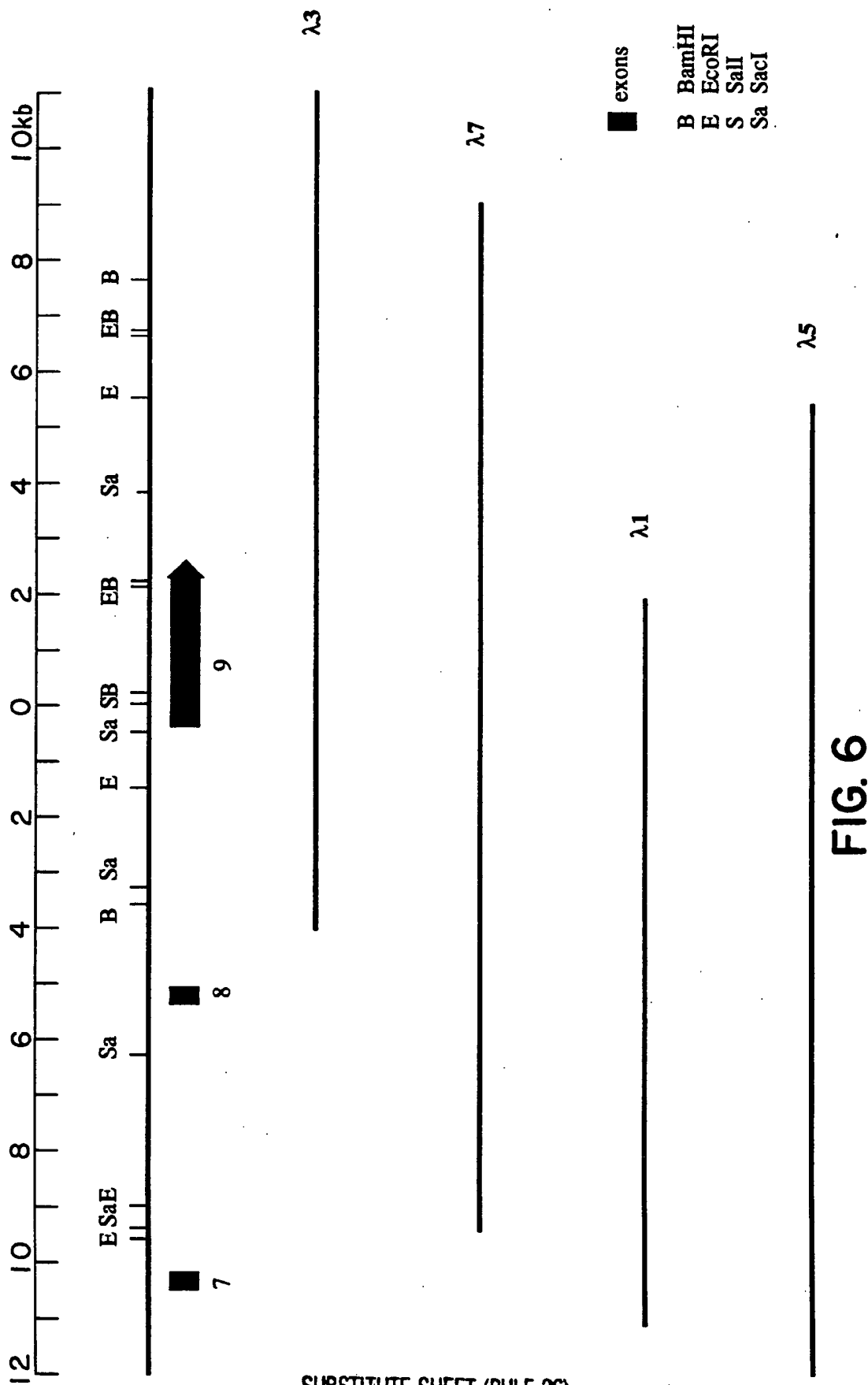
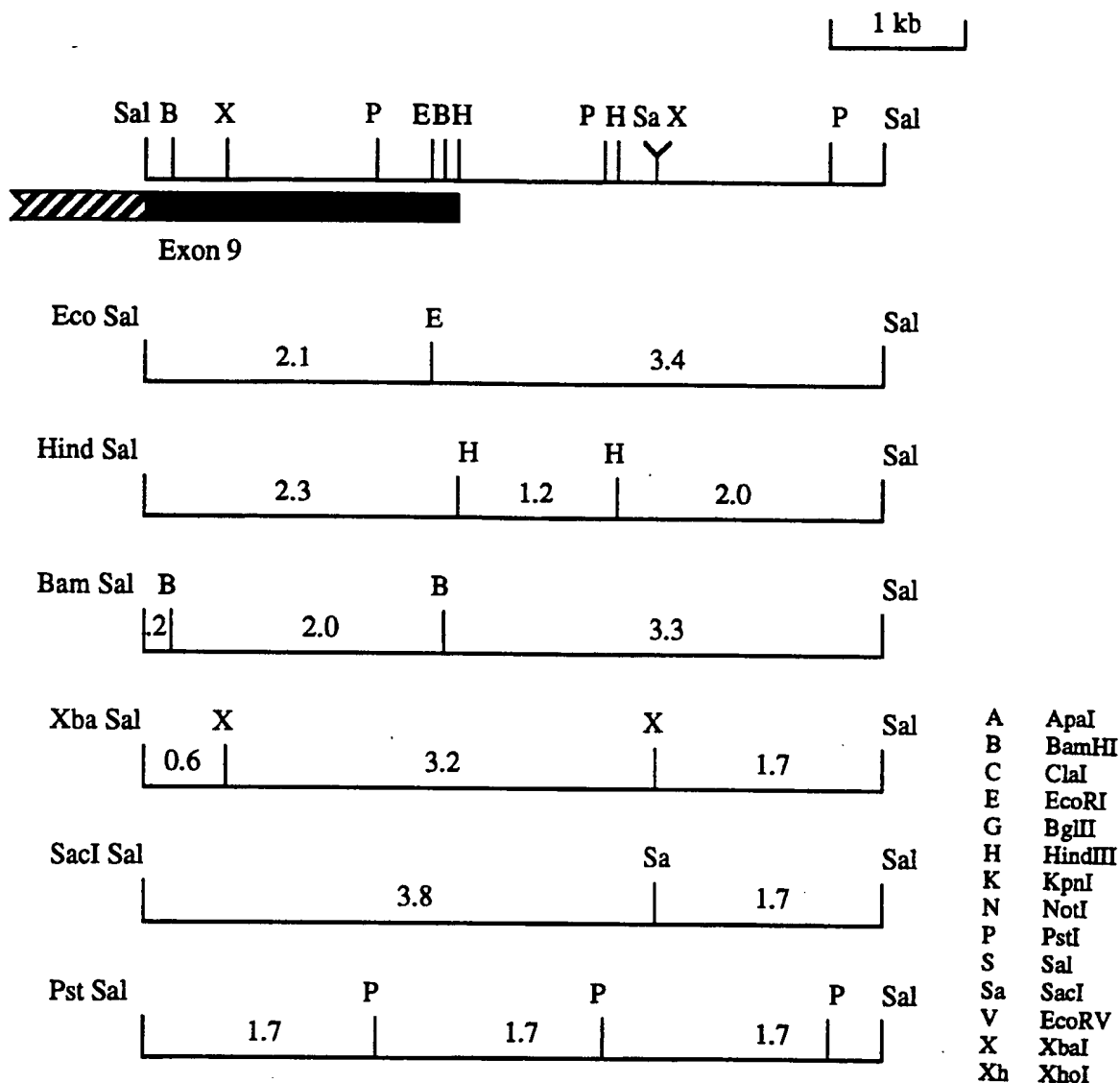


FIG. 6

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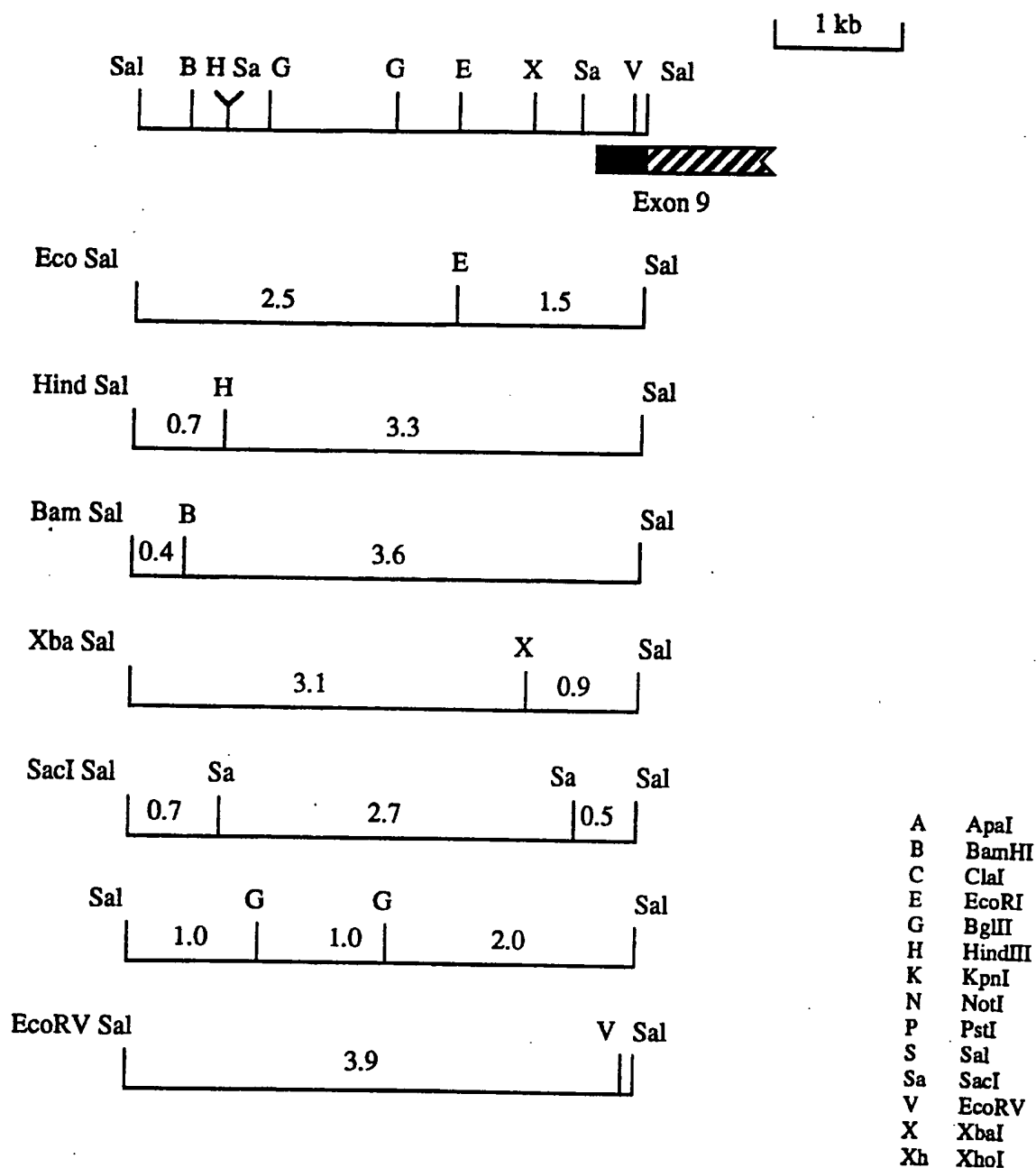
No sites for: BglII, Nde, PvuI, Xho, Kpn, SacII, EcoRV, Sma, Cla, Apa, Not

pBS+KS: .... SacI SacII Not Xba Spe Bam Sma Pst Eco RV Hind Cla Sal Xho Apa Kpn ....



FIG. 7

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No sites for: Nde, PvuI, Xho, Kpn, SacII, Sma, Cla, Apa, Not

Unmapped sites for: Pst, PvuII

pUBS:

.... SacI SacII Not Xba Spe Bam Sma Pst Eco RV Hind Cla Sal Xho Apa Kpn ....

**FIG. 8**

SUBSTITUTE SHEET (RULE 26)



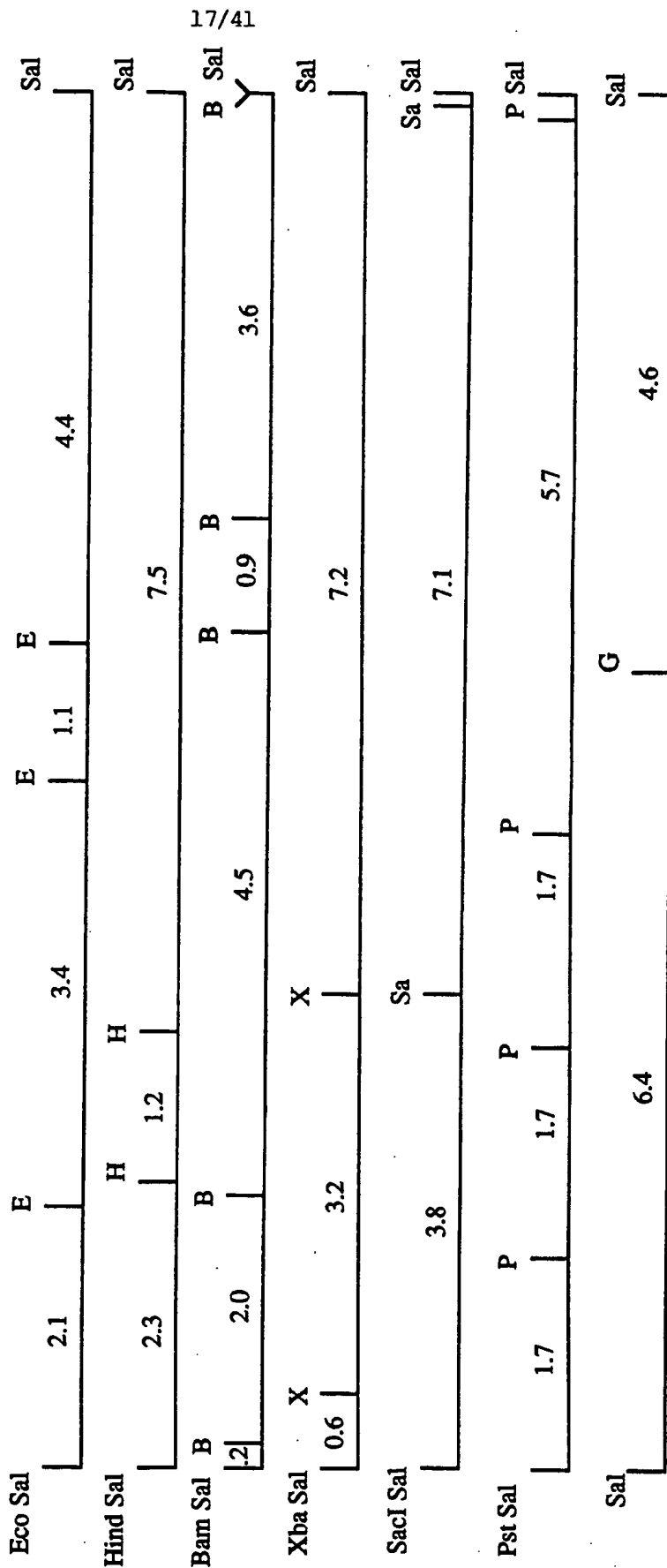
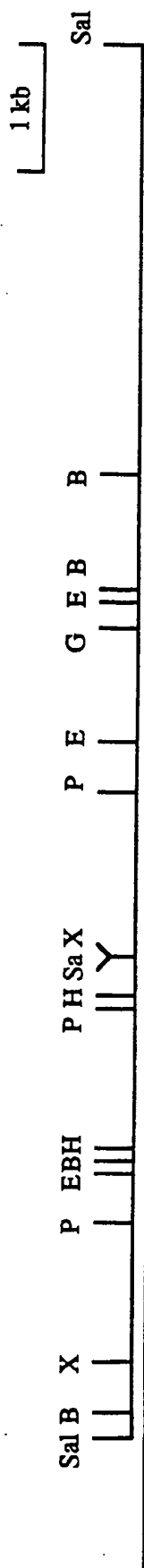


FIG. 9a

A	B	C	E	G	H	K	N	P	S	Sa	V	X	Xh
Apal	BamHI	Clal	EcoRI	BglII	HindIII	KpnI	NotI	PstI	Sal	SacI	EcoRV	XbaI	XhoI

No sites for: Xho,Kpn, SacII, Sma, Cla, EcoRV, Apa, Not, PvuI, Nde

polylinker sites in vector pUBS (pUC19 with polylinker from pBluescript M13+):

.... SacI SacII Not Xba Spe Bam Sma Pst Eco RV Hind Cla Sal Xho Apa Kpn ....



FIG. 9b

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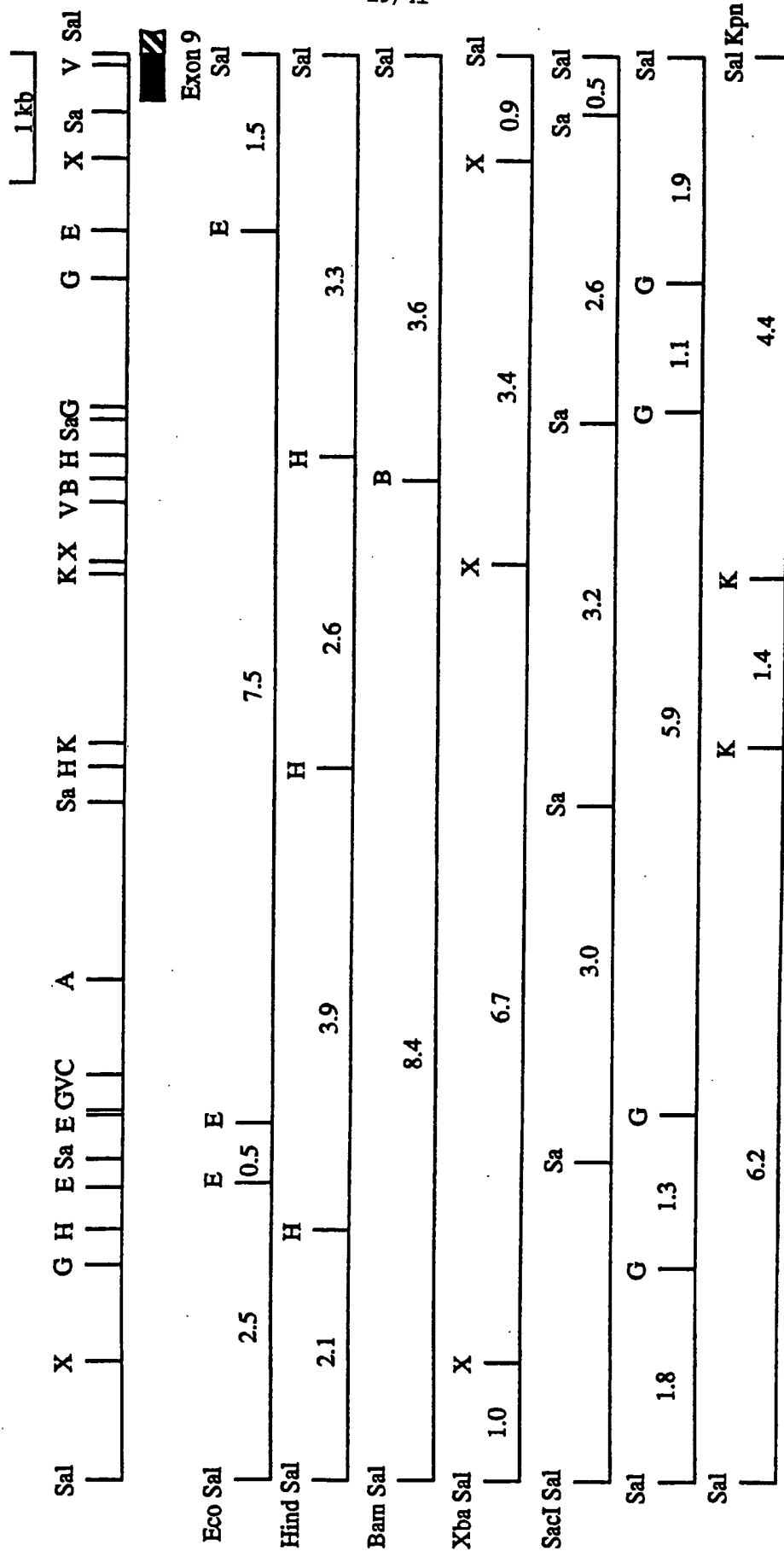


FIG. 10a

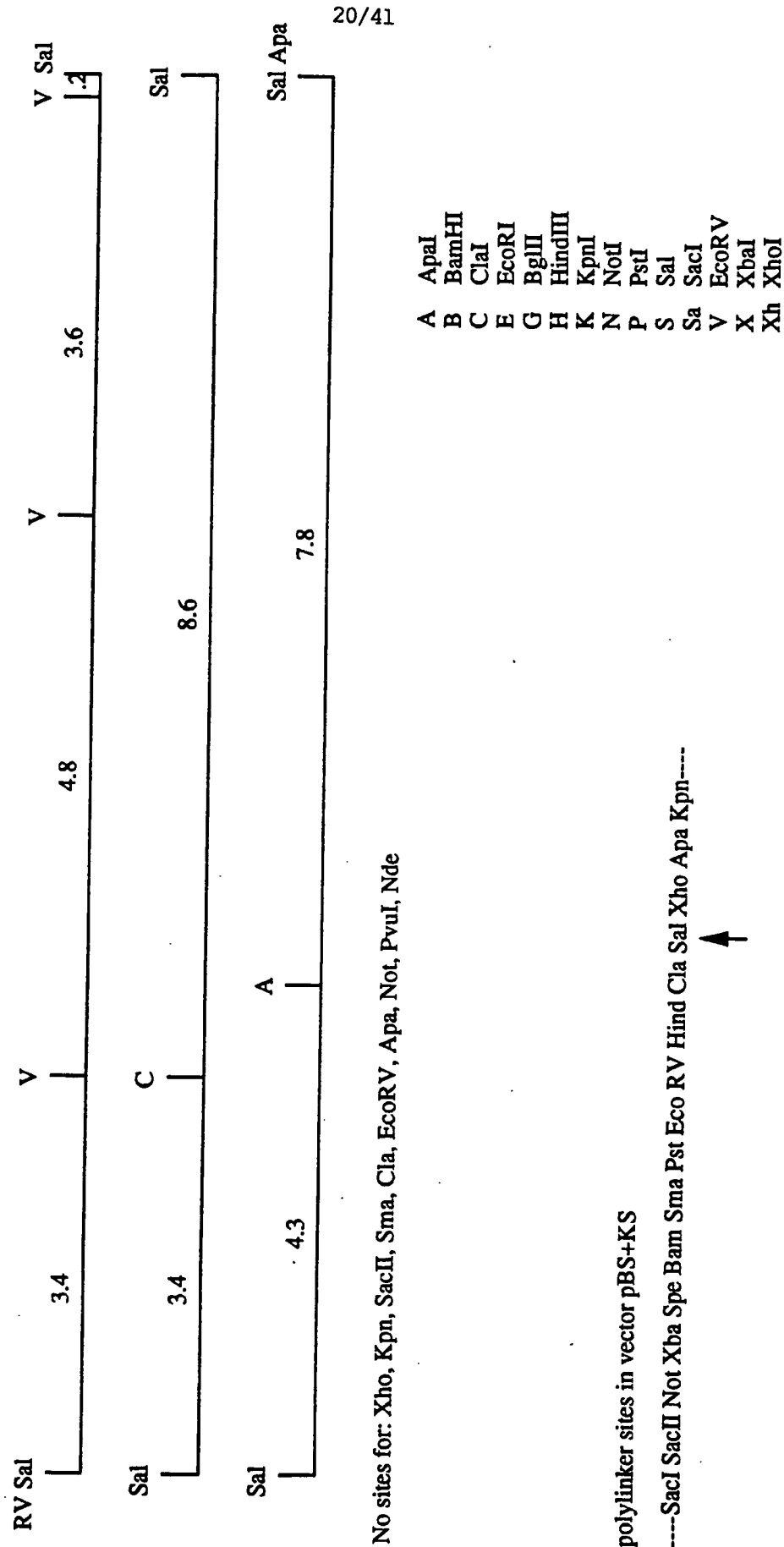


FIG. 10b

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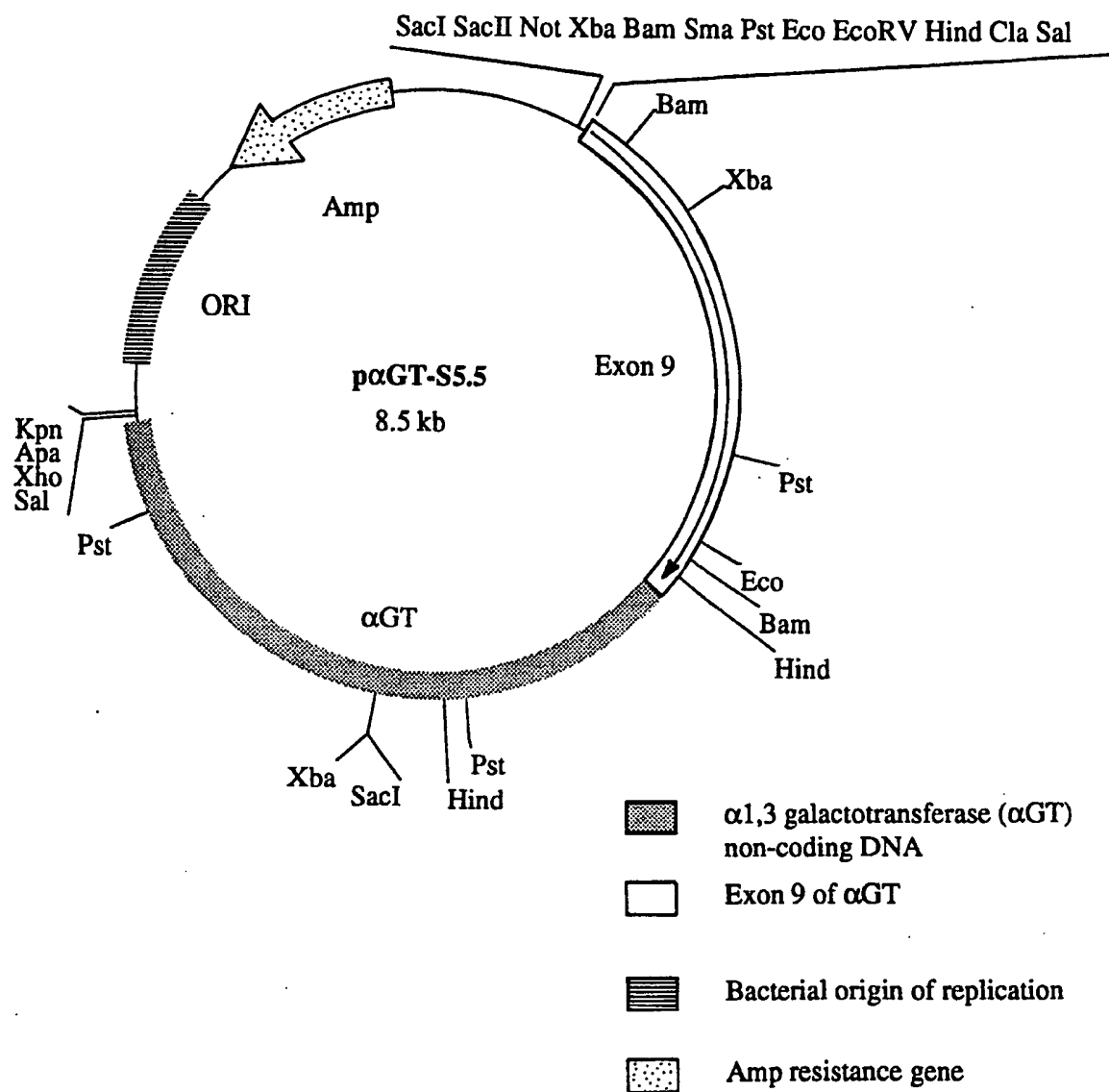


FIG. II

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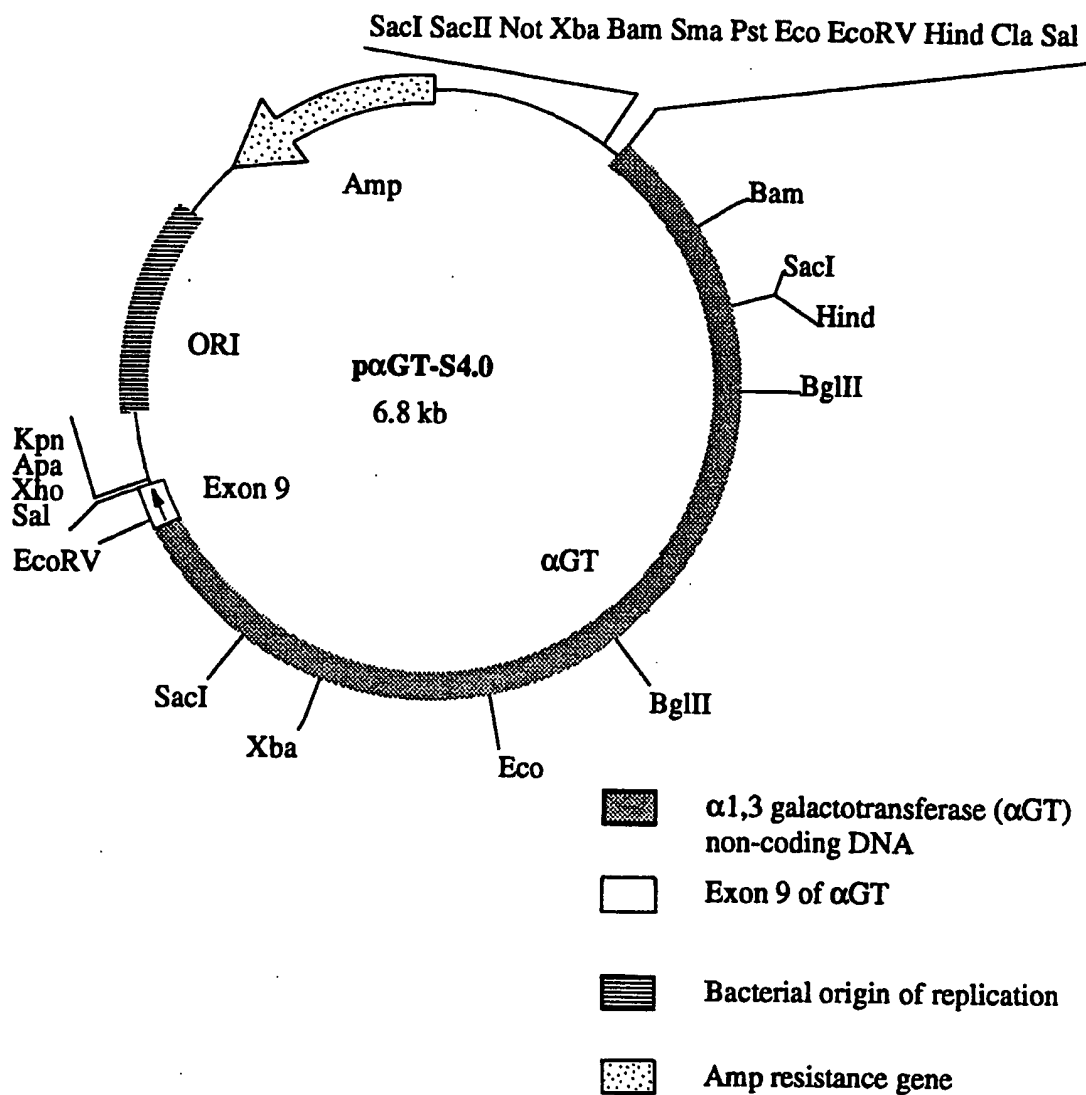
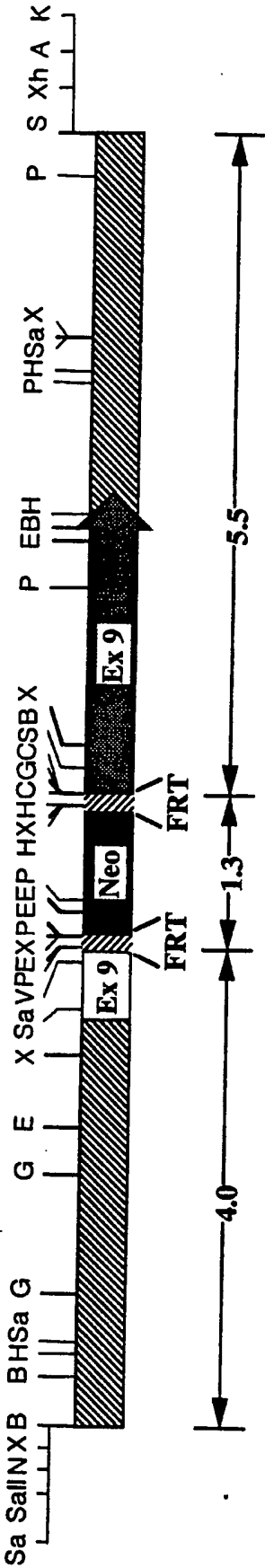


FIG. 12



Region around Cla-Bgl linker

Apal

A

$\alpha$ 1,3 galactotransferase ( $\alpha$ GT)

BamHI

B

non-coding DNA

ClaI

C

Exon 9 of  $\alpha$ GT

EcoRI

E

Neo resistance gene

BglII

G

FRT recombination site

HindIII

H

Cla-Bgl linker

KpnI

K

\*

NotI

N

\*

PstI

P

\*

Sal

S

\*

SacI

Sa

\*

EcoRV

V

\*

XbaI

X

\*

XhoI

Xh

\*

FIG. 13

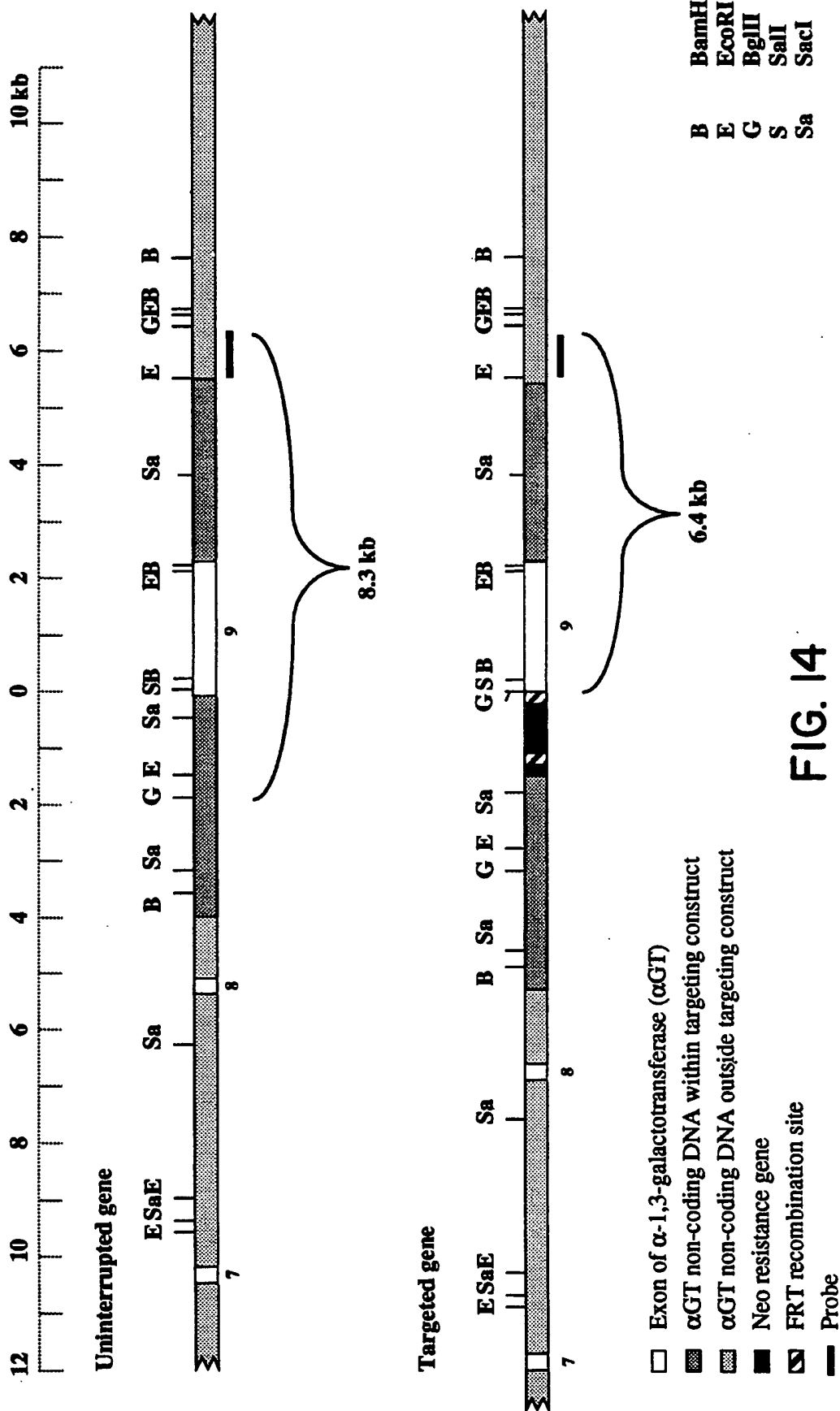


FIG. 14



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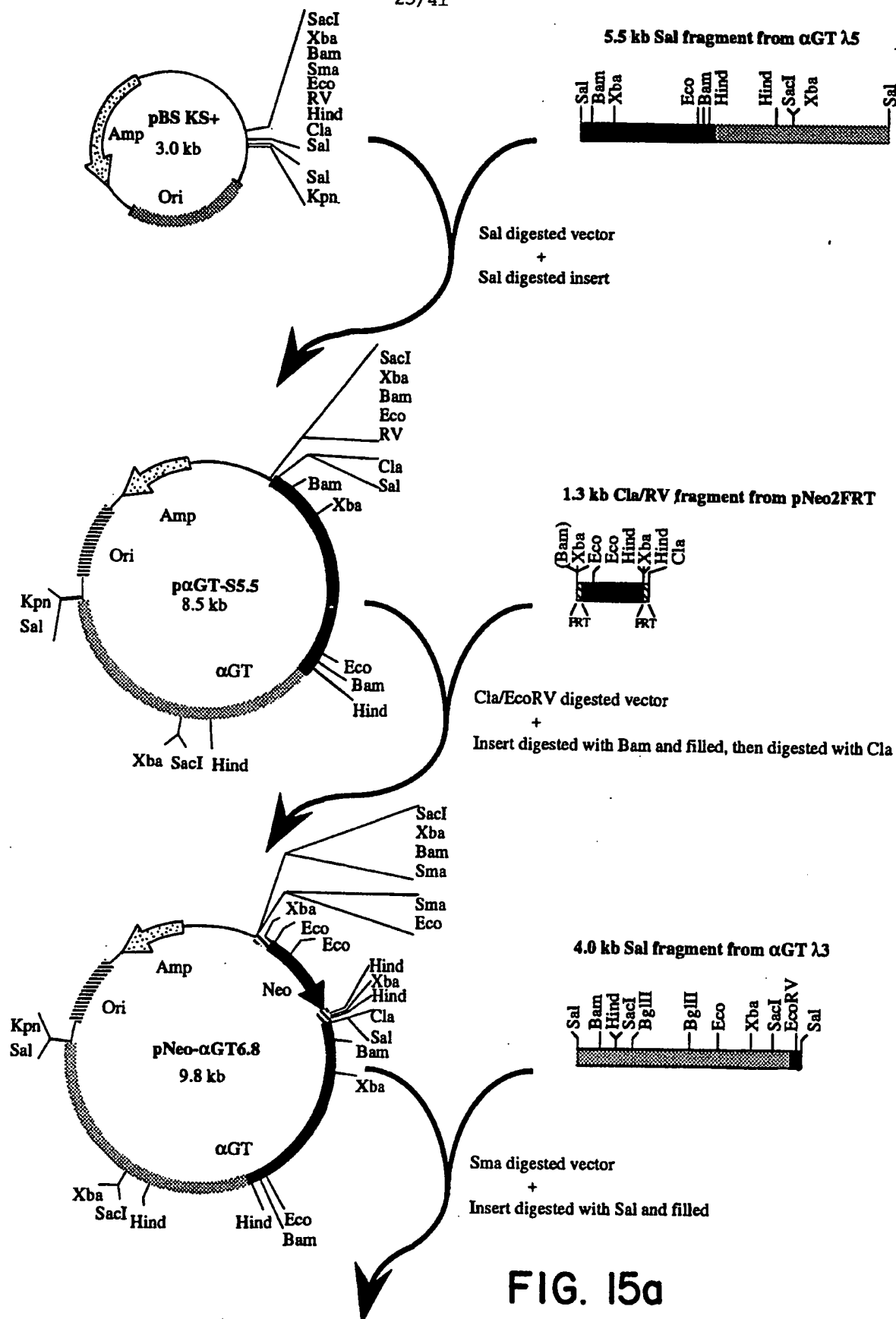


FIG. 15a

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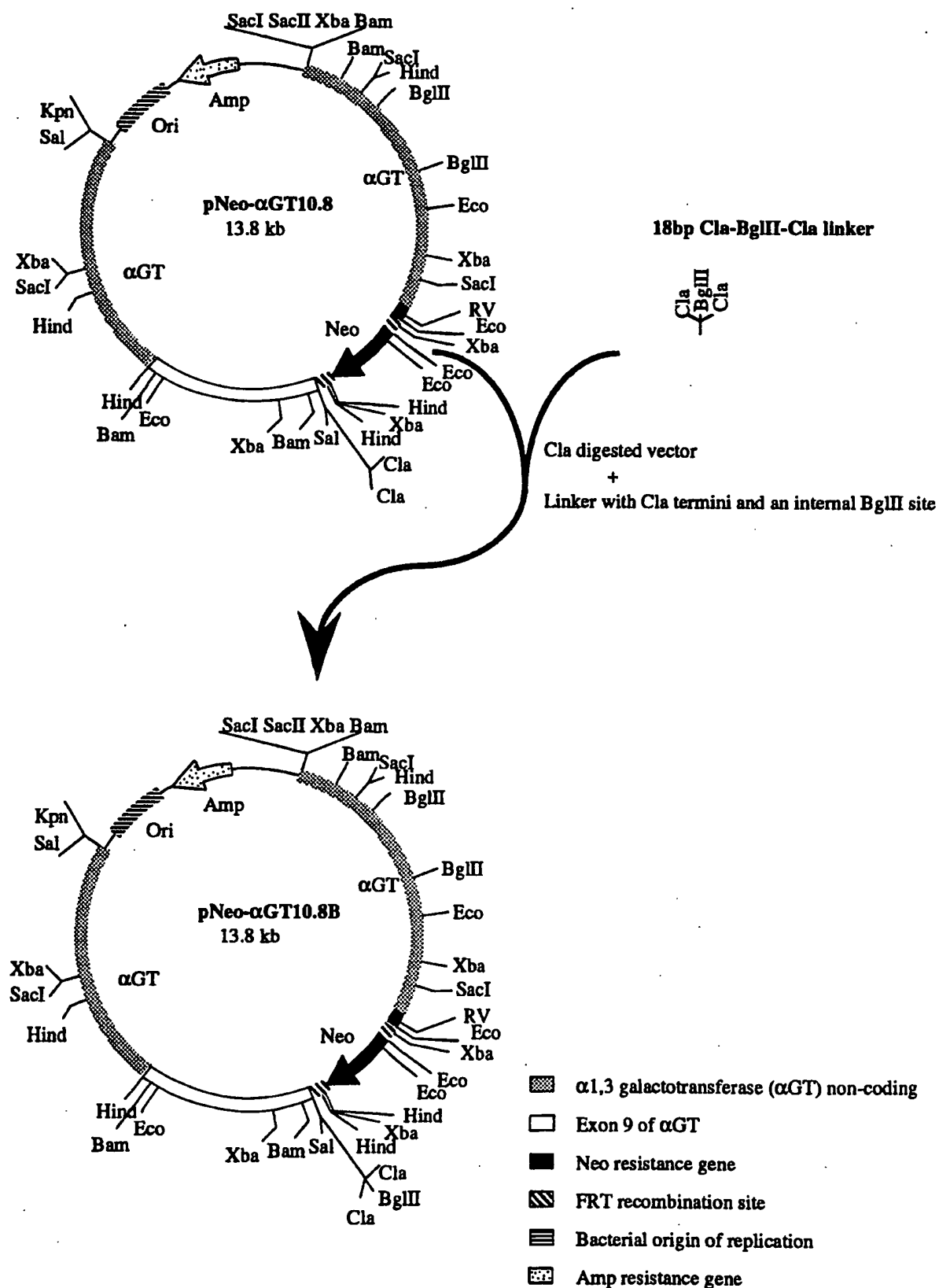


FIG. 15b

SUBSTITUTE SHEET (RULE 26)

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10	20	30	40	50	60
GAGGGCTGCA	GGAATTCGAT	GATCCCCCAG	CTTGAAGTTC	CTATTCCGAA	GTTCCCTATTC
70	80	90	100	110	120
TCTAGAAAGT	ATAGGAACTT	CAAGCTGGGC	TGCAGGAATT	CGATTTCGAGC	AGTGTGGTTT
130	140	150	160	170	180
TGCAAGAGGA	AGCAAAAAGC	CTCTCCACCC	AGGCCTGGAA	TGTTTCCACC	CAATGTCGAG
190	200	210	220	230	240
CAGTGTGGTT	TTGCAAGAGG	AAGCAAAAAG	CCTCTCCACC	CAGGCCTGGA	ATGTTTCCAC
250	260	270	280	290	300
CCAATGTCGA	GCAAACCCCG	CCCAGCGTCT	TGTCATTGGC	GAATTCGAAC	ACGCAGATGC
310	320	330	340	350	360
AGTCGGGGCG	GCGCGGTCCC	AGGTCCACTT	GGCATATTAA	GGTGACGCGT	GTGGCCTCGA
370	380	390	400	410	420
ACACCGAGCG	ACCCTGCAGC	CAATATGGGA	TCGGCCATTG	AACAAGATGG	ATTGCACGCA
430	440	450	460	470	480
GGTTCTCCGG	CCGCTTGGGT	GGAGAGGCTA	TTCGGCTATG	ACTGGGCACA	ACAGACAATC
490	500	510	520	530	540
GGCTGCTCTG	ATGCCGCCGT	GTTCCGGCTG	TCAGCGCAGG	GGCGCCCGGT	TCTTTTGTGTC
550	560	570	580	590	600
AAGACCGACC	TGTCCGGTGC	CCTGAATGAA	CTCCAAGACG	AGGCAGCGCG	GCTATCGTGG
610	620	630	640	650	660
CTGGCCACGA	CGGGCGTTCC	TTGCGCAGCT	GTGCTCGACG	TTGTCACTGA	AGCGGGAAGG
670	680	690	700	710	720
GACTGGCTGC	TATTGGGCGA	AGTGCCGGGG	CAGGATCTCC	TGTCATCTCA	CCTTGCTCCT
730	740	750	760	770	780
GCCGAGAAAG	TATCCATCAT	GGCTGATGCA	ATGCGGCGGC	TGCATACGCT	TGATCCGGCT
790	800	810	820	830	840
ACCTGCCCAT	TCGACCACCA	AGCGAAACAT	CGCATCGAGC	GAGCACGTAC	TCGGATGGAA

FIG. 16a

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850	860	870	880	890	900
GCCGGTCTTG	TCGATCAGGA	TGATCTGGAC	GAAGAGCATC	AGGGGCTCGC	GCCAGCCGAA
910	920	930	940	950	960
CTGTTGCGCA	GGCTCAAGGC	GCGGATGCCC	GACGGCGAGG	ATCTCGTCGT	GACCCATGGC
970	980	990	1000	1010	1020
GATGCCTGCT	TGCCGAATAT	CATGGTGGAA	AATGGCCGCT	TTTCTGGATT	CATCGACTGT
1030	1040	1050	1060	1070	1080
GGCCGGCTGG	GTGTGGCGGA	CCGCTATCAG	GACATAGCGT	TGGCTACCCG	TGATATTGCT
1090	1100	1110	1120	1130	1140
GAAGAGCTTG	GCGGCGAATG	GGCTGACCGC	TTCCTCGTGC	TTTACGGTAT	CGCCGCTCCC
1150	1160	1170	1180	1190	1200
GATTCGCAGC	GCATCGCCTT	CTATCGCCTT	CTTGACGAGT	TCTTCTGAGG	GGATCGGCAA
1210	1220	1230	1240	1250	1260
TAAAAAGACA	GAATAAAACG	CACGGGTGTT	GGGCGTTTGT	TCGGATCATC	AAGCTTGAAG
1270	1280	1290	1300	1310	1320
TTCCTATTCC	GAAGTTCCTA	TTCTCTAGAA	AGTATAGGAA	CTTCAAGCTT	ATCGATGAGT
1330	1340	1350	1360	1370	1380
AGATCTTGAT	CGATACCGTC	.....	.....	.....	.....

Linker sequences : 0-28

FRT : 29-104

Polyoma virus enhancer repeats : 105-249

Herpes Simplex Virus Tyrosine Kinase promoter : 250-385

Neomycin phosphotransferase coding region : 385-1188

Herpes Simplex Virus Tyrosine Kinase PolyA signal : 1189-1249

FRT : 1250-1310

Linker sequences : 1311-1340

**FIG. 16b**

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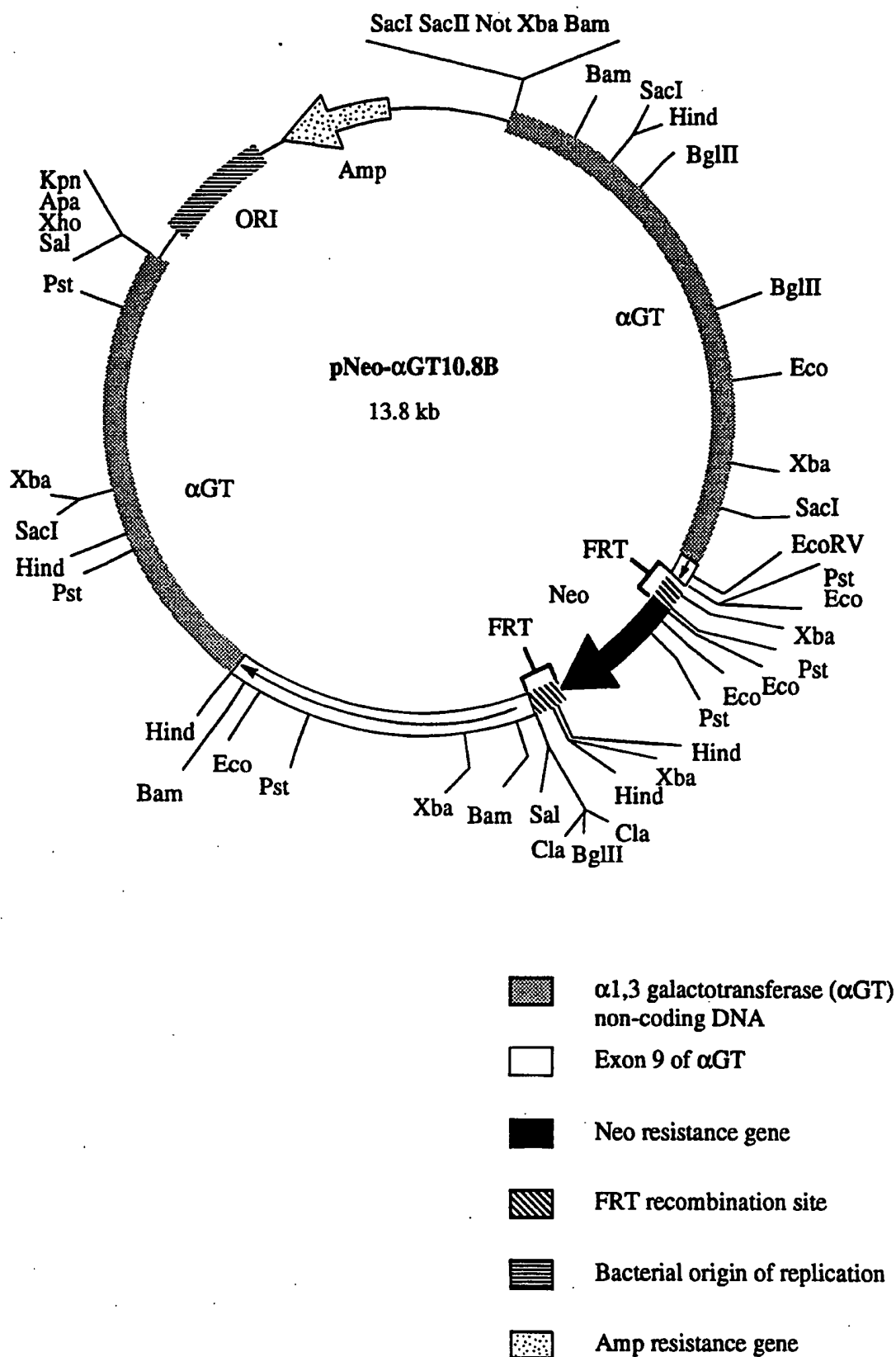


FIG. 17

**SUBSTITUTE SHEET (RULE 26)**

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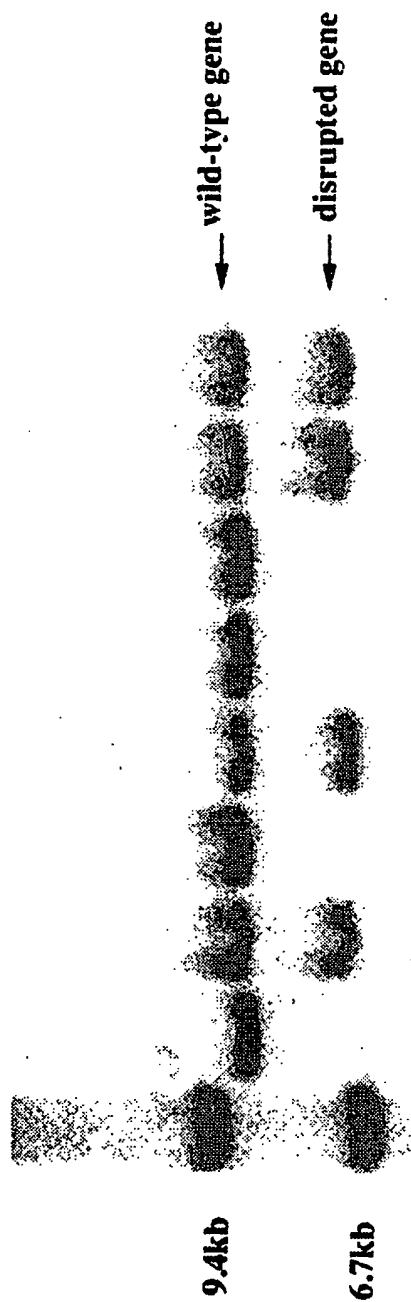


FIG. 18

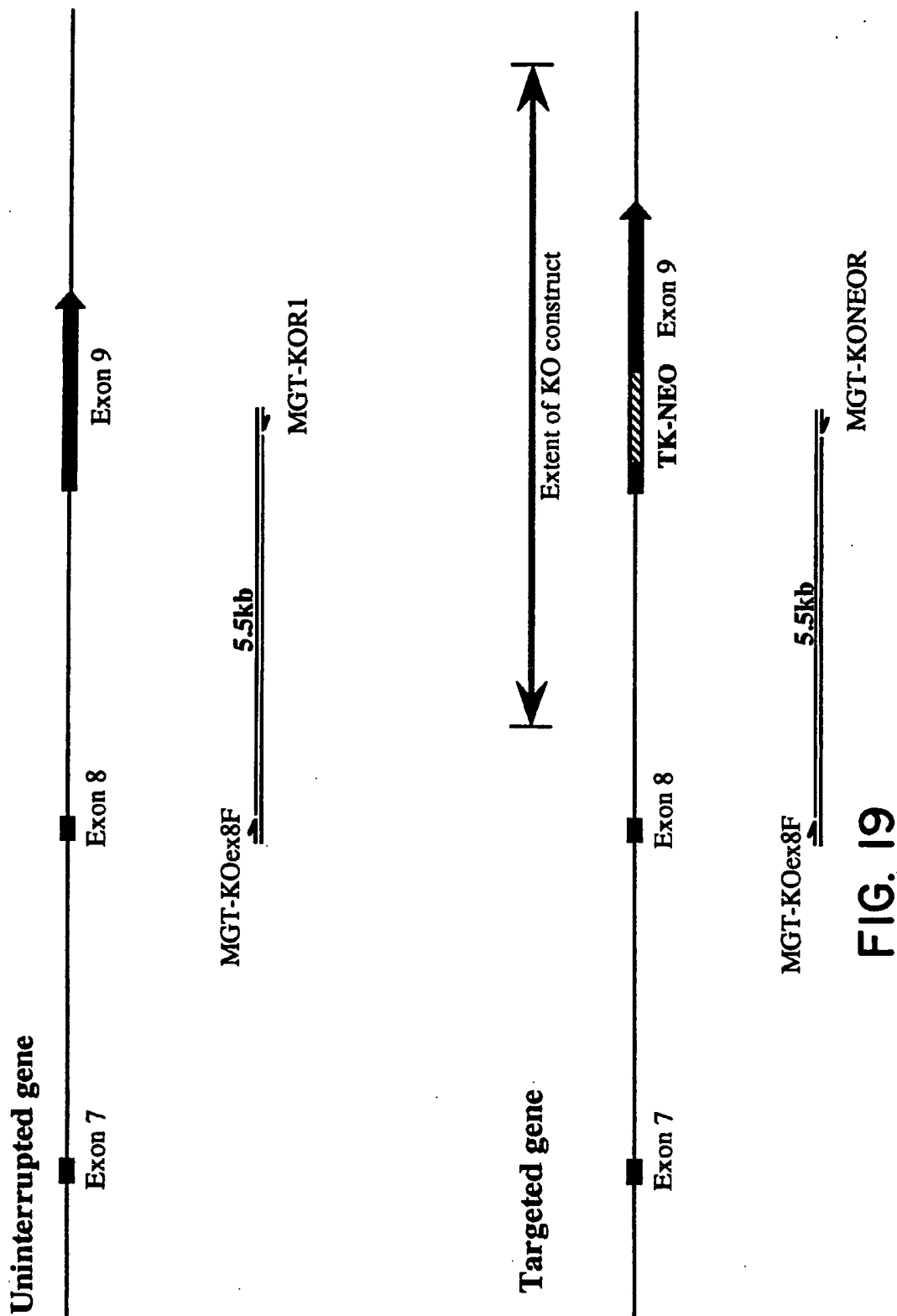
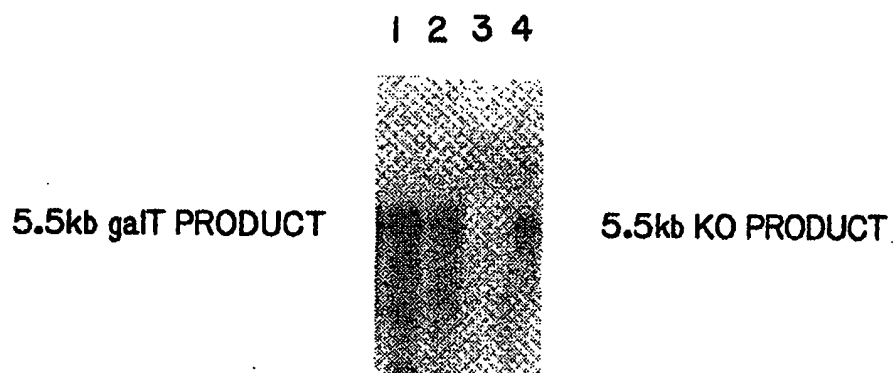


FIG. 19

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1. CBAC TEMPLATE; WILD TYPE PRIMERS
2. 7C2 TEMPLATE; WILD TYPE PRIMERS
3. CBAC TEMPLATE; KO PRIMERS
4. 7C2 TEMPLATE; KO PRIMERS

**FIG. 20**



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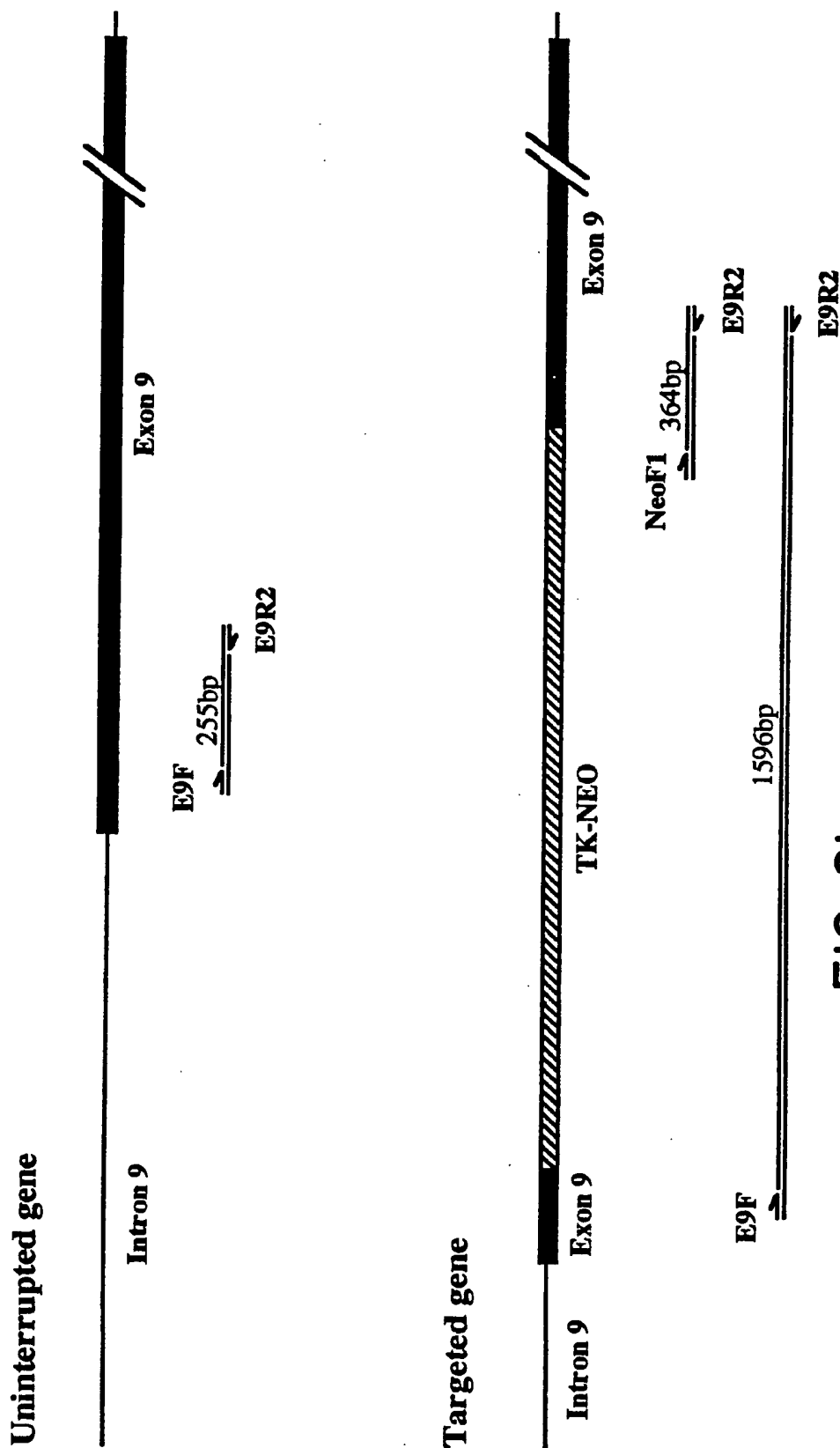


FIG. 2I

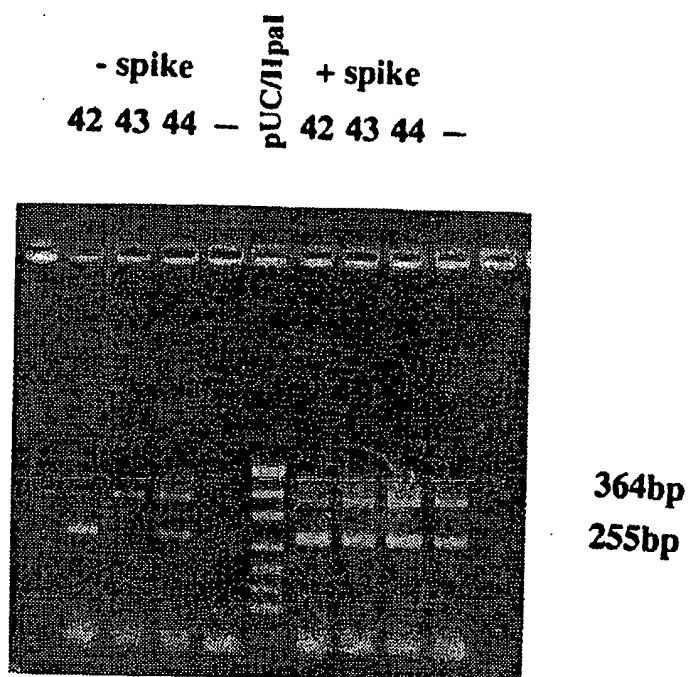
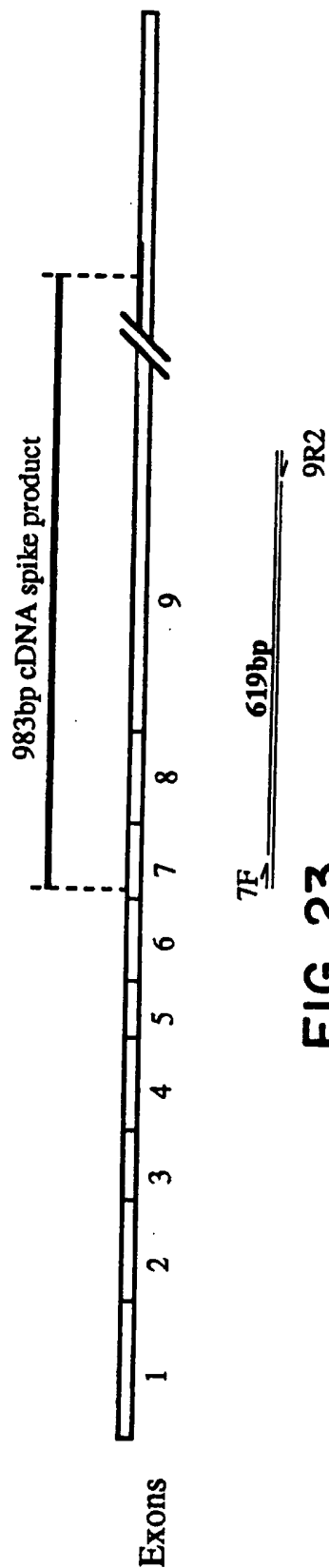


FIG. 22

**Primer binding sites within mouse ferrochelatase cDNA**



**Primer binding sites within mouse  $\alpha$ -1,3-GalT cDNA**



**FIG. 23**

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## i) Ferrochelatase, FC-F/R

M, Marker SPP-I  
 C, MQW control  
 K, KIDNEY  
 H, HEART  
 L, LIVER

FIG. 24a

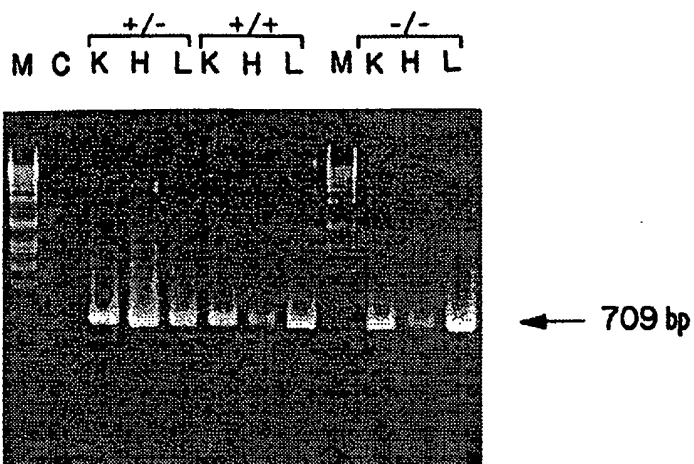
ii)  $\alpha$ -1,3-GT cDNA spike  
+ 7F/9R2 primers

FIG. 24b

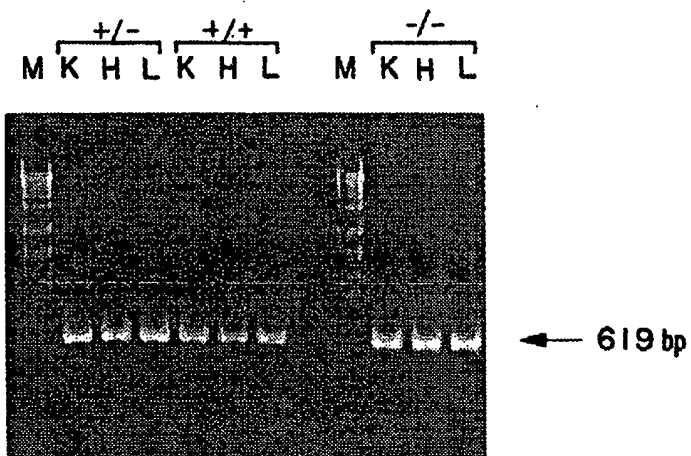
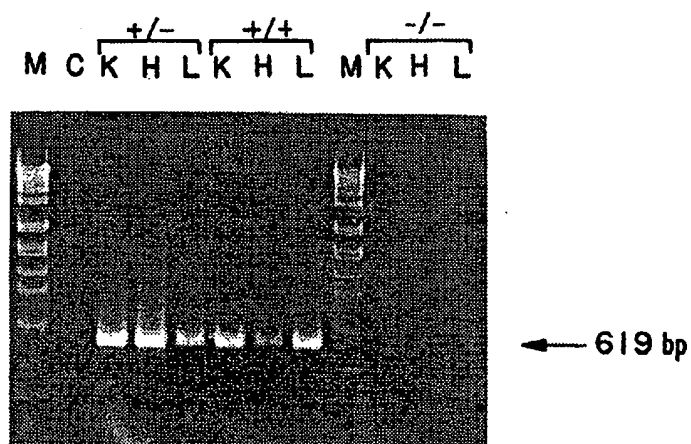
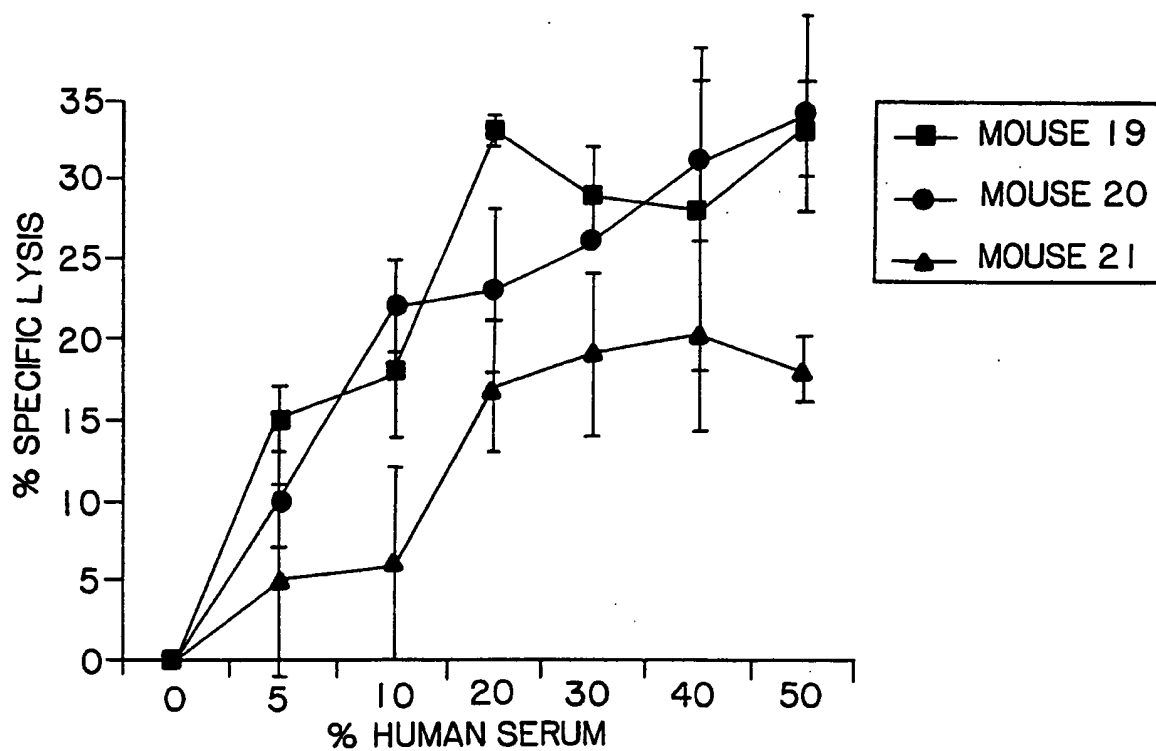
iii)  $\alpha$ -1,3-GT  
7F/9R2 primers

FIG. 24c



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MOUSE 19: WILD TYPE; MOUSE 20: HETEROZYGOTC Gal KO; MOUSE 21:  
HOMOZYGOUS Gal KO

**FIG. 25**

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1	GGAGTCCAGCCCATAATGAAGGTCTTGGCCGCAGGGATTGTGCCCTTACTGCTGCTGGTT	60
61	CTGCACTGGAAACACGGGGCAGGGAGCCCTCTTCCCATCACCCCTGTAAATGCCACCTGT	120
1	MetAsnGlnIleLysAsnGlnLeuAlaGln	10
121	GCCATACGCCACCCATGCCACGGCAACCTCATGAACCAGATCAAGAATCAACTGGCACAG	180
10	LeuAsnGlySerAlaAsnAlaLeuPheIleSerTyrTyrThrAlaGlnGlyGluProPhe	30
181	CTCAATGGCAGCGCCAATGCTCTCTTCATTTCCCTATTACACAGCTCAAGGAGAGCCGTTT	240
30	ProAsnAsnValGluLysLeuCysAlaProAsnMetThrAspPheProSerPheHisGly	50
241	CCCAACAACGTGGAAAAGCTATGTGCGCCTAACATGACAGACTTCCCATCTTTCCATGGC	300
50	AsnGlyThrGluLysThrLysLeuValGluLeuTyrArgMetValAlaTyrLeuSerAla	70
301	AACGGGACAGAGAAGACCAAGTTGGTGGAGCTGTATCGGATGGTTCGCATACCTGAGCGCC	360
70	SerLeuThrAsnIleThrArgAspGlnLysValLeuAsnProThrAlaValSerLeuGln	90
361	TCCCTGACCAATATCACCCGGGACCAGAAGGTCCTGAACCCCACTGCCGTGAGCCTCCAG	420
90	ValLysLeuAsnAlaThrIleAspValMetArgGlyLeuLeuSerAsnValLeuCysArg	110
421	GTCAAGCTCAATGCTACTATAGACGTCATGAGGGGCTCCTCAGCAATGTGCTTTGCCGT	480
110	LeuCysAsnLysTyrArgValGlyHisValAspValProProValProAspHisSerAsp	130
481	CTGTGCAACAAGTACCGTGTGGGCCACGTGGATGTGCCACCTGTCCCCGACCACTCTGAC	540
130	LysGluAlaPheGlnArgLysLysLeuGlyCysGlnLeuLeuGlyThrTyrLysGlnVal	150
541	AAAGAAGCCTTCCAAAGGAAAAAGTTGGGTTGCCAGCTTCTGGGGACATACAAGCAAGTC	600
150	IleSerValValValGlnAlaPhe***	159
601	ATAAGTGTTGGTGGTCCAGGCCTTCTAGAGAGGAGGTCTTGAATGTACCATGGACTGAGGG	660
661	ACCTCAGGAGCAGGATCCGGAGGTGGGGAGGGGGCTCAAATGTGCTGGGGTTTGGGACA	720
721	TTGTTAAATGCAAAACGGGGCTGCTGGCAGACCCCAGGGATTTCAGGTACTCACTGCAC	780
781	TCTGGGCTGGGCCATGATGGAATCTGGCAAAGTTGAACTTCCATAGGCAGAGCTTCTAT	840
841	ACAGCCCAGCACCAGCTAGAAATGGCAATGAGGGTGTGGTCTGAGAGATTTCTGTCTCA	900
901	CTCACTCACTCACTCACTCTCACTCACTCACT.....	

**FIG. 26**

SUBSTITUTE SHEET (RULE 26)

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1 GACCTTTTGCCTTTTCTCTCTCCTGGTGCACCATTTCCTCTCCCTCCCTGAGCCGGAGTT 60  
61 GTGCCCCCTGCTGTTGGTTCTGCACTGGAACATGGGGCGGGGAGCCCCCTCCCCATCACC 120  
1 MetAsnGlnIle 4  
121 CCTGTCAACGCCACCTGTGCCATACGCCACCCATGTCACAACAACCTCATGAACCAGATC 180  
4 ArgSerGlnLeuAlaGlnLeuAsnGlySerAlaAsnAlaLeuPheIleLeuTyrTyrThr 24  
181 AGGAGCCAACTGGCACAGCTCAATGGCAGTGCCAATGCCCTCTTTATTCTCTATTACACA 240  
24 AlaGlnGlyGluProPheProAsnAsnLeuAspLysLeuCysGlyProAsnValThrAsp 44  
241 GCCCAGGGGGAGCCGTTCCCCAACCAACCTGGACAAGCTATGTGGCCCCAACGTGACGGAC 300  
44 PheProProPheHisAlaAsnGlyThrGluLysAlaLysLeuValGluLeuTyrArgIle 64  
301 TTCCCGCCCTTCCACGCCAACGGCACGGAGAAGGCCAAGCTGGTGGAGCTGTACCGCATA 360  
64 ValValTyrLeuGlyThrSerLeuGlyAsnIleThrArgAspGlnLysIleLeuAsnPro 84  
361 GTCGTGTACCTTGGCACCTCCCTGGGCAACATCACCCGGGACCAGAAGATCCTCAACCCC 420  
84 SerAlaLeuSerLeuHisSerLysLeuAsnAlaThrAlaAspIleLeuArgGlyLeuLeu 104  
421 AGTGCCCTCAGCCTCCACAGCAAGCTCAACGCCACCGCCGACATCCTGCGAGGCCTCCTT 480  
104 SerAsnValLeuCysArgLeuCysSerLysTyrHisValGlyHisValAspValThrTyr 124  
481 AGCAACGTGCTGTGCCGCTGTGCAGCAAGTACCACGTGGGCCATGTGGACGTGACCTAC 540  
124 GlyProAspThrSerGlyLysAspValPheGlnLysLysLysLeuGlyCysGlnLeuLeu 144  
541 GGCCCTGACACCTCGGGTAAGGATGTCTTCCAGAAGAAGAAGCTGGGCTGTCAACTCCTG 600  
144 GlyLysTyrLysGlnIleIleAlaValLeuAlaGlnAlaPhe\*\*\* 159  
601 GGGAAGTATAAGCAGATCATCGCCGTGTTGGCCCAGGCCTTCTAGCAGGAGGTCTTGAAG 660  
661 TGTGCTGTGAACCGAGGGATCTCAGGAGTTGGGTCCAGATGTGGGGGCCTGTCCAAGGGT 720  
721 GGCTGGGCCCAGGGCATCGCTAAACCCAAATGGGGGCTGCTGGCTGACCCCGAGGGTGCC 780  
781 TGGCCAGTCCACTCCACTCTGGGCTGGGCTGTGATGAAGCTGAGCAGAGTGGAACCTTCC 840  
841 ATAGGGAGGGAGCTAGAAGAAGGTGCCCCTTCTCTGGGAGATTGTGGACTGGGGAGCGT 900  
901 GGGCTGGACTTCTGCCTCTACTTGTCCCTTTGGCCCCCTTGCTCACTTGTGCAGTGAACA 960  
961 AACTACACAAGTCATCTACAAGAGCCCTGACC.....

FIG. 27

SUBSTITUTE SHEET (RULE 26)

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FIG. 28

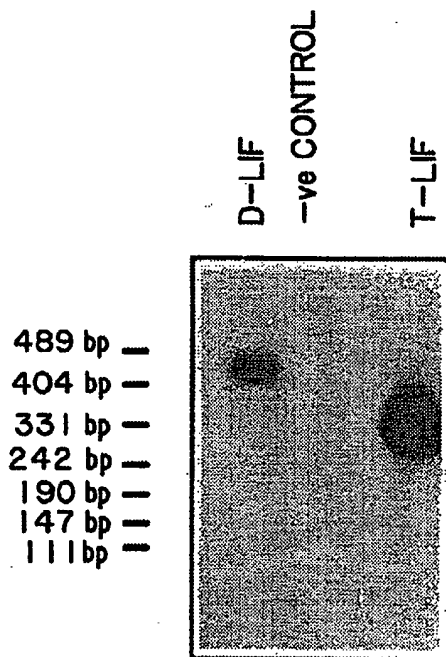
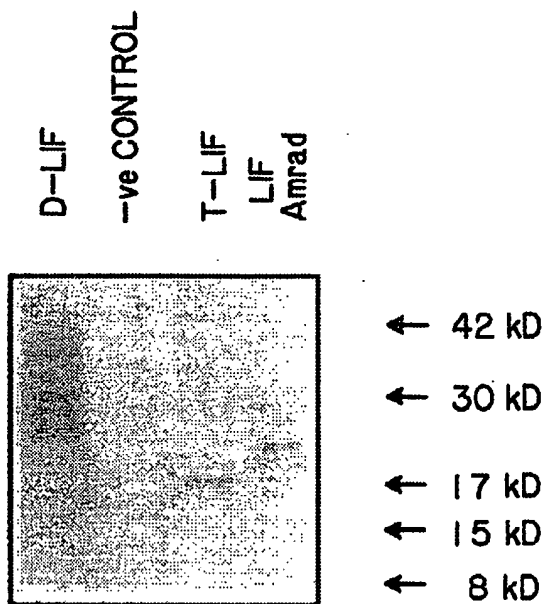
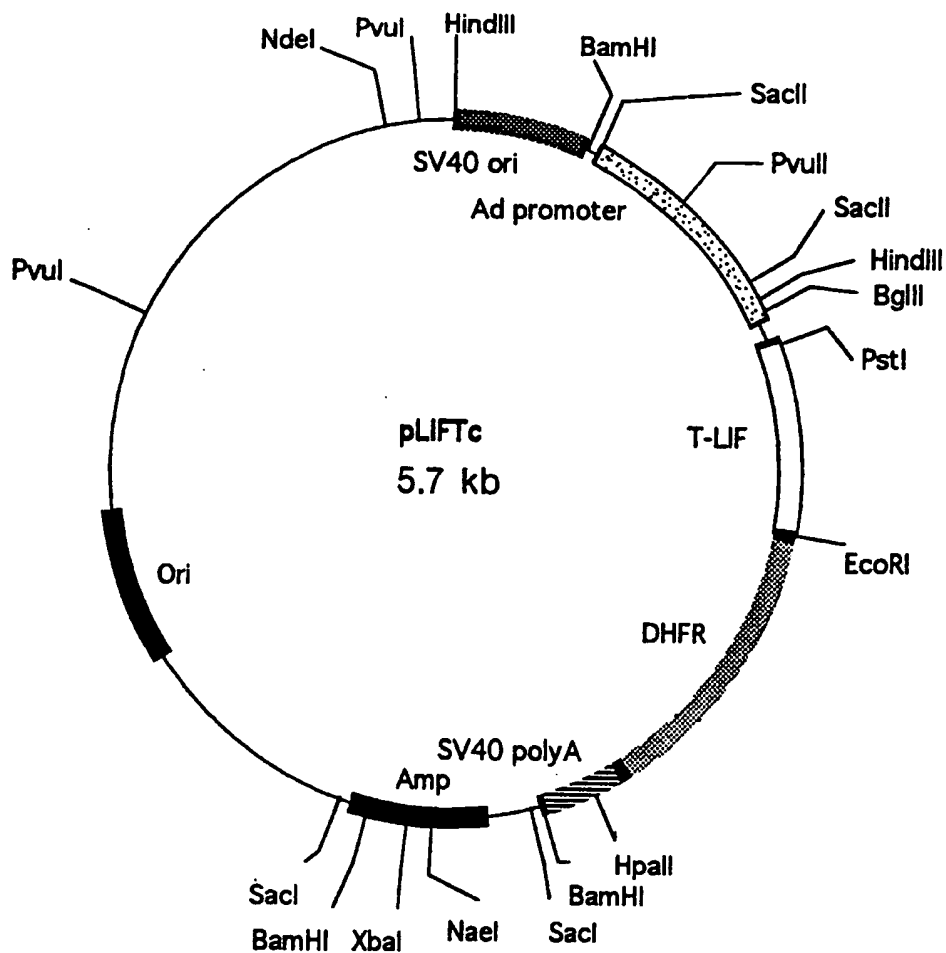


FIG. 30




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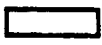
 Dihydrofolate reductase 3' end

 Adenovirus promoter

 SV40 origin of replication

 SV40 PolyA signal

 T-LIF coding region

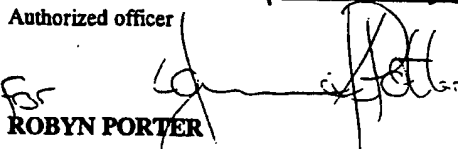
 Bacterial origin of replication

# FIG. 29

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/IB 95/00088

<b>A. CLASSIFICATION OF SUBJECT MATTER</b> Int. Cl. <sup>6</sup> C12N 15/54, 15/19, A61K 31/70, 35/16  According to International Patent Classification (IPC) or to both national classification and IPC					
<b>B. FIELDS SEARCHED</b>  Minimum documentation searched (classification system followed by classification symbols) Electronic databases: WPAT, CASM. Both through QUESTEL ORBIT. Keywords as below.  Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic databases: BIOT, USPM, JAPIO, STN, Medline Embase. Keywords as below. AU IPC C12N 15/54, 15/19.  Electronic data base consulted during the international search (name of data base, and where practicable, search terms used) Keywords for WPAT, USPM, BIOT, JAPIO, CASM. Galactosyltransferase#; Galactosyltransferase#; TOLIF; Leukemia(inhibitor)factor#; IGG; IGM; Human;; Transplant;; Graft;; Reduc;; Deplete#; Less;; Reject;; Hyperacut;; Sensitiv;; Transplant; Transplantation; 03/CC (Last 3 for CASM only) STN Sequences 1. AATGTCAA[AG]GGAA[GA]AGT[GA][GA]T; GCCATTTTGG[GA]GGAAC[AG]CC[CT]AC 2. YYTAQGEPPNNVEKLCAPNM; LGTSLGNITRDQKILNPSALS Keywords for MEDLINE, EMBASE (through DIALOG) Transplant?; Reject?; antial; anti(gal; antigalactos?; anti(galactos; galactos?					
<b>C. DOCUMENTS CONSIDERED TO BE RELEVANT</b>					
<b>Category*</b>	<b>Citation of document, with indication, where appropriate, of the relevant passages</b>	<b>Relevant to Claim No.</b>			
X	M.S. Sandrin et al: "Anti-pig IgM antibodies in human serum react predominantly with Gal (α1-3) Gal epitopes". Proc Natl. Acad. Sci. USA, vol 90, pp 11391-11395, December 1993. See abstract; p 11393: section titled "Human Anti-pig Antibodies React Predominantly with Terminal Galactose Residues"; p 11393, column 2 last line to p 11394 column 2 line 2; p 11394: "Discussion" first paragraph; p 11395, first 2 sentences of 2nd full paragraph and first 10 lines 10 last paragraph.	1-3, 12, 15, 18, 21-26, 28, 29, 31, 38, 39			
<div style="display: flex; justify-content: space-between;"> <div> <input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. </div> <div> <input checked="" type="checkbox"/> See patent family annex. </div> </div>					
<table style="width: 100%; border: none;"> <tr> <td style="width: 30%; vertical-align: top;"> <p>* Special categories of cited documents :</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </td> <td style="width: 10%; vertical-align: top; text-align: center;"> <p>"T"</p> <p>"X"</p> <p>"Y"</p> <p>"&amp;"</p> </td> <td style="width: 60%; vertical-align: top;"> <p>later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>document member of the same patent family</p> </td> </tr> </table>			<p>* Special categories of cited documents :</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p>	<p>"T"</p> <p>"X"</p> <p>"Y"</p> <p>"&amp;"</p>	<p>later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>document member of the same patent family</p>
<p>* Special categories of cited documents :</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p>	<p>"T"</p> <p>"X"</p> <p>"Y"</p> <p>"&amp;"</p>	<p>later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>document member of the same patent family</p>			
Date of the actual completion of the international search 20 June 1995		Date of mailing of the international search report 27 JUNE 1995 (27.06.95)			
Name and mailing address of the ISA/AU AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION PO BOX 200 WODEN ACT 2606 AUSTRALIA  Facsimile No. 06 2853929		Authorized officer  <b>ROBYN PORTER</b>  Telephone No. (06) 2832318			

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/IB 95/00088

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate of the relevant passages	Relevant to Claim No.
P,X	WO 94/21799 (AUSTIN RESEARCH INSTITUTE) 29 September 1994. See entire specification	1-4, 8, 38, 39
X	M.S. Sandrin & I.F.C. McKenzie: "Gal $\alpha$ (1,3)Gal, the Major Xenoantigen(s) Recognised in Pigs by Human Natural Antibodies". Immunological Reviews, 141, 1, pp 169-190, 1994. See page 174, last paragraph, lines 5-8; p 175 lines 4-5; p 176 lines 4-14; p 176, 2nd full paragraph; p 176, line 4 of last paragraph to end of paragraph; p 177, lines 20-30; p 178, Figure 2; p 184, Table III; p 186, lines 14-15 of "Summary".	1, 4-11, 38-43
P,X	WO 94/02616 (THE REGENTS OF THE UNIVERSITY OF MICHIGAN), 3 February 1995. See Sequence ID Numbers 13 and 14.	1
X	K. Gustafsson et al: " $\alpha$ 1,3Galactosyltransferase: A Target for <u>in vivo</u> Genetic Manipulation in Xenotransplantation".	1-3
Y	Immunological Reviews, 141, 1, pp 59-70, 1994. See p 63 lines 7-9; p 68 lines 9-11, 16-24.	4
Y	U. Galili: "Interaction of the natural anti-Gal antibody with $\alpha$ -galactosyl epitopes: a major obstacle for xenotransplantation in humans". Immunology Today, 14, 10, pp 480-482, 1993.	4, 8, 12-31
Y	R.D. Larsen et al: "Isolation of a cDNA encoding a murine UDPgalactose: $\beta$ -D-galactosyl-1,4-N-acetyl-D-glucosaminide $\alpha$ -1,3-galactosyltransferase: Expressin cloning by gene transfer". Proc. Natl. Acad. Sci. USA, 86, pp 8227-8231, November 1989. See entire document but especially abstract; p 8227, column 2, last sentence of Introduction; p 8228, column 2 - "Results"; p 8231, last paragraph.	32-37
Y	F. A. Fletcher et al: "Leukemia Inhibitory Factor Improves Survival of Retroviral Vector-infected Hematopoietic Stem Cells In Vitro, Allowing Efficient Long-term Expression of Vector-encoded Human Adenosine Deaminase In Vivo. "The Journal of Experimental Medicine, 174, 4, pp 837-45, 1991. See Summary; Introduction; p 839, Section of Results titled "Effect of LIF on Tem Cell Survival In Vitro"; p 844, column 2, lines 10-12.	32-37
Y	WO 91/13985 (J. HEATH, A. SMITH & P. RATHJEN), 19 September 1991. See page 1 lines 3-18, page 2 lines 12-14, p 4 line 5 - p 5 line 1, p 5 lines 16-20, p 11 lines 21-26, Examples, Claims 1, 5, 6, 7.	32-37
P,Y	B. B. Samal et al: "High level expression of human leukemia inhibitory factor (LIF) from a synthetic gene in <u>Escherichia coli</u> and the physical and biological characterization of the protein". Biochimica et Biophysica Acta, 1260, pp 27-34, 1995. See entire document, especially Figures 2 and 3.	32-37
Y	T.A. Willson et al: "Cross-species comparison of the sequence of the leukaemia inhibitory factor gene and its protein". European Journal of Biochemistry, 204, 1, pp 21-30, 1992. See entire document, especially p 25 lines 36-39 and 41-57.	32-37
Y	N.M. Gough et al: "Molecular Cloning and expression of the human homologue of the murine gene encoding myeloid leukemia-inhibitory factor". Proc. Natl Acad Sci, 85, pp 2623-2627, April 1988. See entire document especially Figures 3 and 4.	32-37

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate of the relevant passages	Relevant to Claim No.
Y	D.P. Gearing et al: "Complete sequence of murine myeloid leukaemia inhibitory factor "(LIF)". Nucleic Acids Research, 16, 20, p 9857, 1988. See entire document.	32-37
Y	J.F. Moreau et al: "Leukaemia inhibitory factor is identical to the myeloid growth factor human interleukin for DA cells". Nature, 336, pp 690-692, 15 December 1988. See abstract, Figure 1.	32-37
Y	WO 88/07548 (AMRAD CORPORATION LIMITED) 6 October 1988. See page 1 lines 2-5, claims 1-3, 7, 8, 13-17, 34, 35.	32-37
Y	D.P. Gearing et al: "Production of Leukemia Inhibitory Factor in <u>Escherichia coli</u> by a Novel Procedure and Its Use in Maintaining Embryonic Stem Cells in Culture". Bio/Technology, 7, pp 1157-1161, November 1989. See abstract, p 1157, column 2, lines 2-10, 42-45, p 1159, column 2, lines 15 - end of paragraph.	32-37
Y	T. Yamamori et al: "The Cholinergic Neuronal Differentiation Factor from Heart Cells Is Identical to Leukemia Inhibitory Factor". Science, 246, pp 1412-1416, 15 December 1989. See entire document.	32-37
Y	D.G. Lowe et al: "Genomic Cloning and Heterologous Expression of Human Differentiation-Stimulating Factor". DNA, 8, 5, pp 351-359, 1989. See whole document especially abstract; p 352, column 1, lines 1-8, 21-22; p 352 "Materials and Methods".	32-37
Y	J. Stahl et al: "Structural Organization of the Genes for Murine and Human Leukemia Inhibitory Factor", Journal Biol Chem, 265, 15, pp 8833-41, 1990. See entire document.	32-37
P,X	H.A. Vaughan et al: "Gal $\alpha$ (1,3)Gal is the major xenoepitope expressed on pig endothelial cells recognized by naturally occurring cytotoxic human antibodies". Transplantation, 58, 8, pp 879-882, 1994. See p 879, column 2 last 5 lines to p 880, column 1, last 5 lines of 'Materials and Methods'; p 880, column 1, last 10 lines 10 column 2, end of section; p 882, column 1, 11th line from bottom to end.	38-39
X	WO 93/16729 (BIOTRANSPLANT, INC) 2 September 1993. See page 3 lines 1-14 and 23-33; page 6 last 4 lines - page 7 line 2; page 14, section titled "IgM Depleting Technique"; claims 1, 8, 10.	40-41
X	WO 92/07581 (AUTOIMMUNE, INC) 14 May 1992. See claim 11.	40
P,Y	D. Latinne et al: "Depletion of IgM xenoreactive Natural Antibodies by Injection of anti- $\mu$ Monoclonal Antibodies". Immunological Reviews, 141, 1, pp 94-125, 1994. See page 98, full paragraph; page 99 lines 11-13; page 102, new section, lines 3-12 and 14-16; page 106 lines 3-11; page 110, sentence beginning on 3rd last line to page 111, line 2; page 115, lines 3-11; page 116 sentence on last line - page 117 line 1, page 117, lines 3-21 of "Conclusion"; page 118, paragraph 2 lines 3-5.	40-43

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/IB 95/00088

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate of the relevant passages	Relevant to Claim No.
P,Y	M. Soares et al: "In Vivo IgM depletion by Anti- $\mu$ Monoclonal Antibody Therapy". Transplantation, 57, 7, pp 1003-1009, 1994. See abstract lines 23-27; p 1003 column 2, lines 1-10 and 33-39; p 1004, column 1, lines 6-9; column 2 lines 3-5; p 1006, figure 3, column 2, lines 4-8, p 1007, column 2 lines 2-5 and 11-28; p 1009, column 1, lines 20-24.	40, 41
X	M. Soares et al: "In Vivo Depletion of Xenoreactive Antibodies with an Anti- $\mu$ Monoclonal Antibody". Transplantation, 56, 6, pp 1427-1433, 1993. See abstract, 2nd sentence, p 1428, column 1, last full sentence, p 1429, paragraph spanning columns 1 and 2, Figure 1; p 1429, column 2, lines 16-43, p 1430, figures 3 and 4; p 1431, 1st sentence of 'DISCUSSION'.	40,41
X	L. Gambiez et al: "The Role of Natural IgM in the Hyperacute Rejection of Discordant Heart Xenografts". Transplantation, 54, 4, pp 577-583, 1992. See last paragraph of abstract; p 577, column 2, last sentence of introduction; p 579, column 1 line 19 - column 2 line 14; p 580, column 1 line 22-23, 29-30 and column 2 lines 14-20; p 582, column 1, last full sentence.	40, 41
P,A	R. Oriol et al: "Monomorphic and polymorphic carbohydrate antigens on pig tissues: implications for organ xenotransplantation in the pig-to-human model". Transplant International, 7, 6, pp 405-413, 1994.	1-7, 12, 15-18, 21-26, 28, 29, 31
A	WO 90/08188 (AMRAD CORPORATION LIMITED) 26 July 1990.	32-37
A	EP 235805 (THE ROYAL FREE HOSPITAL SCHOOL OF MEDICINE) 9 September 1987.	
A	G. Hale et al: "Removal of T Cells From Bone Marrow for Transplantation: A Monoclonal Antilymphocyte Antibody That Fixes Human Complement". Blood, 62, 4, pp 873-882, 1983.	

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/IB 95/00088

## Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international search report has not established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 18, 26 and 29  
because they relate to subject matter not required to be searched by this Authority, namely:

They include humans within their scope. However, the claims were searched as if they specifically excluded humans ie "... non-human mammals ...".

2. ☐ Claim Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Claims 1 to 31;  
Claims 32 to 37;  
Claims 38 to 39; and  
Claims 40 to 45

As reasoned on "extra" sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically  
claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims;  
it is covered by claims Nos.:

## Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☒ No protest accompanied the payment of additional search fees.

## Box II (continued)

The inventors have established that most xenoantibodies are directed to a terminal  $\alpha$  1,3 galactose linkage (termed the GAL epitope) residue on the cell surface of graft tissues.

Using this observation they have developed several strategies for limiting graft rejection.

Invention 1 as defined in claims 1 to 31 is to eliminate the GAL epitope on the donor organ (as described on page 15) by interfering with the expression of the enzyme responsible for forming the  $\alpha$  1,3 galactose linkage.

Invention 2 as defined in claims 38 to 39 is to block the circulating GAL antibodies in the recipient by IV administration of an  $\alpha$  1,3 galactose which will bind to the antibody. Other sugars can also block the antibody when administered intravenously (see pages 21 to 22).

Invention 3 as defined in claims 40 to 45 is to non-specifically deplete the recipient of total IgM prior to transplantation to reduce the acute phase of Ig response (see page 22).

Invention 4 as defined in claims 32 to 37 is directed to a novel LIF. LIF has been identified as a suitable differentiation inhibiting factor for growth of ES cells having an inactivated  $\alpha$  1,3 GalT gene for the development of a transgenic animal (see page 30).

Since inventions 2 and 3 are directed at either blocking or depleting the recipient's Ig levels and invention 1 is directed at eliminating the GAL epitope on the donor organs, the international application does not comply with the requirements of unity of invention because the inventions defined do not share a 'technical relationship' and thus these inventions do not relate to one invention or to a single inventive concept.

Further, since invention 1 is directed at eliminating the GAL epitope on the donor organs and invention 4 is directed to an LIF, there is also no 'technical relationship' between these 2 inventions and consequently the international application does not relate to one invention or to a single inventive concept.

### Information on patent family members

**PCT/TB 95/00088**

Patent Document Cited in Search Report				Patent Family Member			
WO	9421799	AU	62792/94				
WO	9402616	EP	654082	IL	106416	US	5324663
WO	9113985	EP	518933	GB	9004890	JP	5504955
WO	8807548	AT	102991	AU	15907/88	DK	4831/89
		EP	285448	FI	894613	HK	336/95
		HU	51330	IL	85961	NO	885339
		NO	950509	NZ	224105	PT	87133
		ZA	8802277	US	5187077		
WO	9316729	AU	37796/93				
WO	9207581	AU	89426/91	BR	9107055	CA	2093513
		EP	555413	HU	63957	IL	99864
		NO	931536				
WO	9008188	AU	48356/90	CA	2045126	EP	453453
		NZ	232072	US	5418159		
EP	235805	AU	69691/87	DK	1098/87	EP	235805
		FI	870916	JP	62281824	NO	870866
		ZA	8701562				

END OF ANNEX



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